The Importance of JNK in Ageing Nerve Growth Factor-Responsive Sympathetic Neurons

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Declaration

I, Isa Guha confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Abstract

In ageing, sub-populations of nerve growth factor (NGF)-responsive neurons are susceptible to neurodegeneration. Whereas aged sympathetic neurons lose their survival dependence on NGF, the NGF precursor, proNGF, has been found to be neurotoxic in these cells and this is mediated by p75-neurotrophin receptor ($p75^{NTR}$) and sortilin. However, the intracellular pathway and mechanisms involved downstream require further investigation.

As developing sympathetic neurons are NGF-dependent for survival, apoptosis has been observed in neonatal superior cervical ganglion (SCG), following NGF withdrawal, which is facilitated by $p75^{NTR}$ but attenuated by c-Jun N-terminal kinase (JNK) inhibition. The p75 neurotrophin receptor-interacting protein (NRAGE) has also been implicated in $p75^{NTR}$-mediated cell death in NGF-responsive neurons. While plenty of research has focused on sympathetic neurons in development, this study sought to examine the effect of JNK and NRAGE inhibition in cultured SCG neurons and glia from aged and young adult mice.

Through the use of pan-JNK inhibitors and AEG3482, which indirectly inhibits JNK via HSP90, the survival of young adult and aged NGF-treated cultured sympathetic neurons was found to be unaffected, but the formation of neurites and glial processes was restricted. Furthermore, using isoform-specific JNK inhibitors, results were consistent with JNK3 having a major role in this process. The survival and neurite outgrowth of NGF-treated cultured aged SCG neurons, subjected to siRNA knockdown of NRAGE, were both unaffected.

Interestingly, both AEG3482 and NRAGE siRNA rescued the proNGF-mediated killing of aged SCG neurons. In addition, similar to NGF-treated cells, proNGF-mediated neurite outgrowth of aged SCG neurons was restricted during JNK inhibition but not following NRAGE knockdown in cell culture. In conclusion, while NRAGE is involved in proNGF-mediated neurotoxicity, JNK is required for both the proNGF-mediated cell death, and neuritogenesis, of SCG neurons and glia from aged mice and these findings have implications for therapeutic intervention.

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Abbreviations

AD	Alzheimers Disease
AL	ad libitum
AP-1	Activator protein -1
ATF3	Activating transcription factor 3
Akt	Protein kinase B
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BFCN	Basal forebrain cholinergic neurons
BSA	Bovine serum albumin
CNS	Central nervous system
CREB protein	cAMP response element binding
DCX	Doublecortin
DMEM	Dulbecco's modified Eagle's medium
DRG	Dorsal root ganglion
DsRNA	Double-stranded RNA
DTT	Dithiothreitol
ERK	Extracellular signal-related kinases
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
IHC	Immunohistochemistry
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
LpL	Lipoprotein Lipase
MAP1B	microtubule assembly
MAPK	Mitogen-activated protein kinase
MKK	Mitogen-activated protein kinase kinase
MMP	matrix metalloprotease
mNGF	mature NGF
NBM	Nucleus basalis of Meynert
NGF	Nerve growth factor
NRAGE	Neurotrophin receptor interacting MAGE homolog
NRH2	Neurotrophin receptor homolog 2
NRIF	Neurotrophin receptor interacting factor
NT-3	Neurotrophin-3
OCT	Optimum cutting temperature
PBS	Phosphate buffered saline
Pen-1	Penetratin-1
PI3K	Phosphatidylinositol 3-kinase
PFA	Paraformaldehyde
PNS	Peripheral nervous system

Abbreviations

RGC	Retinal ganglion cells
RNAi	RNA interference
RNAP	RNA polymerase
RNAse	Ribonuclease
RT	Room temperature
RT-PCR	Reverse Transcription polymerase chain reaction
SAPK	Stress –activated protein kinase
SCG	Superior cervical ganglion
siRNA	Small interfering RNA
SDS	Special diet services
SMAC	Second mitochondria-derived activator of caspases
TBS	Tris buffered saline
TNFα	Tumor necrosis factor alpha
Trk	Tyrosine kinase receptor

Chapter 1
- INTRODUCTION -

1.1 Introduction

Ageing involves the accumulation of deleterious changes in cells and tissues over time, which increases the risk of disease and death. While the average life expectancy has improved, the consequence of an ageing global population is that dementia and other neurodegenerative diseases are fast becoming a major health problem - the knock on effect resulting in increased staff numbers for diagnosis and treatment, but also the huge challenges placed on families, both psychologically and financially. Normal ageing is a challenge to every living organism and what drives the process is still incompletely understood.

There is extensive variation in ageing and, in humans, differences can be determined by nutrition, lifestyle, environment and genetic predisposition (Busse 1987). Phenotypically, the most noticeable changes in neurological function are movement disorders. Among the elderly, poor posture and rigidity of joints can lead to gait abnormality in acute cases (Rubino 1993). The senses are also affected with frequent reports of visual impairment and severe hearing loss (Schneider et al., 2011). In the brain, the delay of central processing times and gathering of new information results in memory loss and other cognitive dysfunctions (Drag and Bieliauskas 2010), while additional changes in the body include a decline in renal function (Zhou et al., 2008) and respiratory performance (Janssens et al., 1999), besides an increase in systolic blood pressure (Tobin and Snyder 1984). Another characteristic of ageing is the atrophy of tissues and organs (Maynard et al., 2015); muscle deterioration is prevalent (Mitchell et al., 2012; Walston 2012) and can severely impact bodily functions.

Growing old is inevitable but ways and means of preserving and lengthening the quality of life are much desired. To fully address the issues, it is essential to understand the basis of ageing-associated decline at the molecular, functional and organismal levels. Central to this is the loss of irreplaceable cells. Biochemical events that promote cell death are initiated when cells fail to respond adaptively to age-related increases in oxidative, metabolic or ionic stress, resulting in an excessive collection of damaged proteins, DNA and membranes. While active regulation is enforced to combat cell atrophy, the breakdown or failure of this process increases the chance of degeneration, thus highlighting the importance of survival mechanisms in aged cells. To complicate matters, cells in adult life are much more diverse than in early development, with extensive crosstalk between signalling pathways, making it

considerably more difficult to dissect the molecular components involved in these pathways.

In the nervous system, cells of all regions are at risk of degeneration; however, there is substantial variation in the rate of decline and the primary locations for damage. Essentially, physical and molecular characteristics, functional properties and location can all determine the fate of neurons and their axons (Morrison et al., 1998). Larger neurons with myelinated axons that extend relatively long distances, from one region of the nervous system to another, are generally more vulnerable e.g. from peripheral neurons to their targets or from central neurons to peripheral targets. High-energy requirement, reliance on axonal transport for trophic support, a large cell surface area exposing cells to toxins, and impaired signal transduction are all contributing factors to this increased susceptibility (Schmidt et al., 1990; Cleveland and Rothstein 2001; Sieradzan and Mann 2001; Cookson 2005; Moore et al., 2005).

While the central nervous system (CNS) struggles to repair itself after injury, the peripheral nervous system (PNS) is well-equipped to regenerate and re-innervate neuronal networks (Aguayo et al., 1991), although this process slows with ageing. Survival in the PNS is then dependent on how effective the recovery operations are in these less functional systems (Verdu and Navarro 1995; Kerezoudi and Thomas 1999; Verdu et al., 2000).

A key regulator of survival, growth and repair is neurotrophic signalling. While neurotrophins are implicated in the age-dependent cell death of neurons (Jansen et al., 2007; Al-Shawi et al., 2008), they also assist with nerve regeneration (Lindsay 1988; Andrews and Cowen 1994b; Gavazzi 1995; Lykissas et al., 2007; Ma et al., 2013), and are used therapeutically in neurodegenerative conditions (Isaacson et al., 1990; Apfel 1999; Siegel and Chauhan 2000; Ross 2004; Aloe et al., 2012). The complexity of the signalling pathway is an important component of neural plasticity, which involves the modification of neural pathways, and is prompted by changes in behaviour, environment and neural processes, or as a result of injury. This is evident at all stages of life, whereby certain characteristic expressions, i.e. the localisation and availability of proteins and receptors, are altered to suit the ever-changing environment.

For instance, mature neurons undergo continual adaptation in response to physiological demands that are quite different from those in early stages. During

embryogenesis, the formation of neuronal networks is crucial, with the number of developing neurons being calibrated to establish an optimally wired nervous system (Oppenheim 1991). As a result, the cell-death pathway is at liberty to remove any unwanted cells, often in large numbers. An important part of this process is to establish a retrograde flow of neurotrophins, from the target cell into the nerve terminal and up the axon to the cell body (Ginty and Segal 2002). Neurons that are unable to manage this transportation are subjected to degeneration and removed from the system. This provides a 'survival of the fittest' type scenario whereby the end result is a matched network of neurons and their target cells (Davies 2003; Buss et al., 2006). Once the retrograde flow of neurotrophins is established, it must proceed for the entire lifetime of the neuron to maintain a functional differentiated state (Barde 1989). As the role of mature cells is to execute vital neuronal functions, the neuronal status shifts from apoptotic vulnerability to survival.

Although the importance of neurotrophins is well documented, knowledge about the mechanisms and pathways involved in ageing require further attention. It is clear that aged neurons become less responsive to mature neurotrophins (Uchida and Tomonaga 1987; Cowen et al., 1996; Orike et al., 2001b). However, while aged neurons are not dependent on these factors for survival, they still require mature neurotrophins for neurite outgrowth (Orike et al., 2001a; Orike et al., 2001b). More importantly, reports in the last 15 years have identified a role for pro-neurotrophins in regulating survival in ageing (Al-Shawi et al., 2008), in response to injury (Harrington et al., 2004; Arnett et al., 2007) and disease (Fahnestock et al., 2004).

This study aims to further characterise the different components of these survival pathways, but also to observe their role during neuronal repair in a sub-population of aged neurotrophic-responsive neurons in the PNS.

1.2 Peripheral Vulnerability – the sympathetic nervous system

The interactions between peripheral sympathetic neurons and their targets have been a major focus for understanding specific mechanisms underlying neuronal atrophy in aged organisms, and molecular events involved in neuronal survival, axon growth, and dendrite arborisation (Schmidt et al., 1990; Cowen and Gavazzi 1998). The sympathetic system is part of the autonomic division, which controls motor outputs of the PNS. Autonomic ganglia innervate practically all body organs and regulate involuntary functions e.g. heart beat, blood pressure, digestion, glandular secretions and smooth muscle movements.

Sympathetic ganglia receive preganglionic inputs from nerve fibres emerging from the thoraco-lumbar regions of the spinal cord and consist of two major groups: paravertebral ganglia (e.g. superior cervical ganglia (SCG)), which are arranged as a chain along the spinal column, and prevertebral ganglia (e.g. celiac ganglia), located near large blood vessels in the abdomen. The various types of neurons in sympathetic ganglia have been thoroughly characterised (McLachlan 1995; Ribeiro et al., 2004; Li and Horn 2006)

Atrophy is common in the sympathetic innervation of numerous peripheral targets in ageing (Schmidt et al., 1990), and degeneration has been observed in SCG axons associated with the cerebral blood vessels (CV) (Cowen and Thrasivoulou 1990; Andrews et al., 1994), pineal gland (Kuchel et al., 1999), and the submandibular gland (Andrews et al., 1996). Further still is the selective vulnerability of specific groups of neurons, as not all axonal projections from the SCG show atrophy e.g. the sympathetic innervation of aged iris is not significantly affected and SCG iris-projecting neurons undergo continued growth throughout ageing (Santer 1991; Andrews et al., 1996).

Of course atrophy is not just restricted to projections from the SCG, and is observed in other sympathetic ganglia, both cervical (Bruzzone et al., 2003) and celiac (Bellinger et al., 1992). However, for the purpose of this study, SCG will remain the primary focus. Previous studies of SCG neuron, glial cell morphology and function have been investigated *in vivo* (Hedger and Webber 1976) and *in vitro* (Tropea et al., 1988). Age-related changes have also been previously described (Roivainen and Koistinaho 1996).

1.3 Neurotrophic signalling

The first neurotrophin to be identified was nerve growth factor (NGF). Discovered by Rita Levi-Montalcini and Stanley Cohen in the 1950s, NGF was established as a target-derived trophic agent in the PNS (Levi-Montalcini and Angeletti 1968; Thoenen et al., 1971), and a promoter of neuronal survival, particularly in peripheral sympathetic ganglia (Levi-Montalcini 1987). In developing sympathetic neurons, NGF was shown to influence size (Snider 1988), axonal arborisation (Saffran and Crutcher 1990), neurotransmitter synthesis (Thoenen et al., 1971) and preganglionic innervation (Purves and Nja 1976). Trophic effects were later observed in the CNS (Seiler and Schwab 1984), mostly promoting survival and preserving the phenotype of cholinergic neurons (Batchelor et al., 1989; Ebendal 1989; Schweitzer 1989; Sofroniew et al., 2001). Other well-recognised members of the neurotrophin family are brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Maisonpierre et al., 1990) and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Ibanez 1995). Their roles in axonal growth and regeneration (Lykissas et al., 2007) and neuronal survival (Barde 1994; Lu et al., 2005) have been reviewed.

Synthesis of neurotrophins principally takes place in peripheral tissues or target neurons that are in contact with axons of neurotrophin-sensitive neurons, usually at extensive distances from the cell body. In the periphery, the tissue sources of neurotrophins are typically non-neuronal cells such as muscle and glands, whereas in the CNS, primary synthesis occurs in neurons (Thoenen 1995).

To deliver trophic effects in the PNS, neurotrophins bind to receptor complexes on nerve endings, which are internalised and retrogradely transported, from distal axons to neuronal cell bodies. The internalised ligand-receptor complex, is incorporated into a signalling endosome and transported along microtubules, using dynein as a motor protein (Zweifel et al., 2005), transmitting signals to mediate survival, growth and gene expression. The retrograde signalling of NGF was first observed by Ian Hendry in SCG targets of the iris (Hendry et al., 1974). Studies have since shown that the initial phases of axonal outgrowth from peripheral ganglia and extension along intermediate targets are NGF-independent (Fagan et al., 1996; Glebova and Ginty 2004; Wickramasinghe et al., 2008), and target-derived NGF is critical for axonal extension and arborisation only after the axons have reached their final destinations.

Mature NGF (mNGF) is translated from a single coding exon, and is synthesised as a larger pro-form (proNGF) of ~27-34kDa, which is cleaved by furin or other pro-protein convertases in the endoplasmic reticulum (ER) and Golgi to produce the C-terminal mature NGF (mNGF) form of 12-13kDa (Suter et al., 1991; Seidah et al., 1996; Lee et al., 2001). *In vivo*, NGF swiftly associates to form a non-covalent dimer of 26.5kDa (Bothwell and Shooter 1977; Narhi et al., 1993). These and higher molecular weight glycosylated NGF precursors have been identified within rodents, both *in vitro* and *in vivo* (Berger and Shooter 1977; Dicou et al., 1986; Edwards et al., 1988; Lakshmanan et al., 1988; Bresnahan et al., 1990; Dicou 1992; Seidah et al., 1996; Reinshagen et al., 2000), and are highly homologous to human NGF (Ullrich et al., 1983).

While NGF is produced and transported from peripheral targets and non-neuronal cells, there is a basal level of production within sympathetic neurons. Hasan et al., (2003) reported that NGF mRNA is expressed in nearly half of the neuronal SCG population at embryonic day 17, rising to over 90% in the early postnatal period, before receding in adult neurons. Further studies in neonatal neurons, using metabolic labelling and immunoprecipitation of NGF-immunoreactive proteins, found no detection of mNGF. This was combined with the detection of high molecular weight NGF isoforms in neonatal ganglion homogenates, suggesting that proNGF is predominantly secreted at this stage and an autocrine loop may exist.

Following synthesis in the ER, pro-neurotrophins undergo folding and sorting into the constitutive (vesicles spontaneously fuse with the plasma membrane to release their protein contents into the extracellular space) or regulated secretory pathway (extracellular signals trigger the fusion of vesicles) before transportation to the correct subcellular compartment. A large number of non-neuronal cells, such as smooth muscle cells, fibroblasts and glia, may not express molecular components of the regulated secretory pathway and, therefore, secrete neurotrophins only constitutively. In neurons, although regulated secretion is prevalent, the bulk of proNGF is secreted constitutively (Lu et al., 2005).

An important role for the pro-domain of proNGF is to ensure correct folding, processing and secretion of mNGF (Suter et al., 1991; Rattenholl et al., 2001), including sorting into constitutive or regulatory secretory pathways. There are three dibasic processing sites in the pro-domain which are targeted for cleavage in the trans-Golgi

network by furins or other pro-protein convertases (Greene et al., 1968; Edwards et al., 1988; Seidah et al., 1996). Proteolysis may also occur extracellularly via plasmins or matrix metalloproteases (MMPs) (Leone et al., 2005; Paggi et al., 2006; De Stefano et al., 2007).

Detailed discussions of the neurotrophin family, including the structure and molecular evolution have been published (Barde 1994). Pleiotropic actions of each of the four mammalian neurotrophins are mediated through selective binding to one or more of their cognate receptor tyrosine kinases, members of the tropomyosin-related kinase (Trk) family (**Fig 1.1**). NT-3 binds to all three Trk receptors (Lamballe et al., 1991; Squinto et al., 1991; Ip et al., 1993), while BDNF and NT-4/5 preferentially bind to TrkB (Klein et al., 1991b; Squinto et al., 1991). In sympathetic systems, practically all neurons require NGF and preferred receptor, TrkA, for survival, axon growth and target innervation (Levi-Montalcini 1987; Edwards et al., 1989; Kaplan et al., 1991; Klein et al., 1991a; Smeyne et al., 1994; Hassankhani et al., 1995; Seidah et al., 1996; Glebova and Ginty 2004; Kuruvilla et al., 2004).

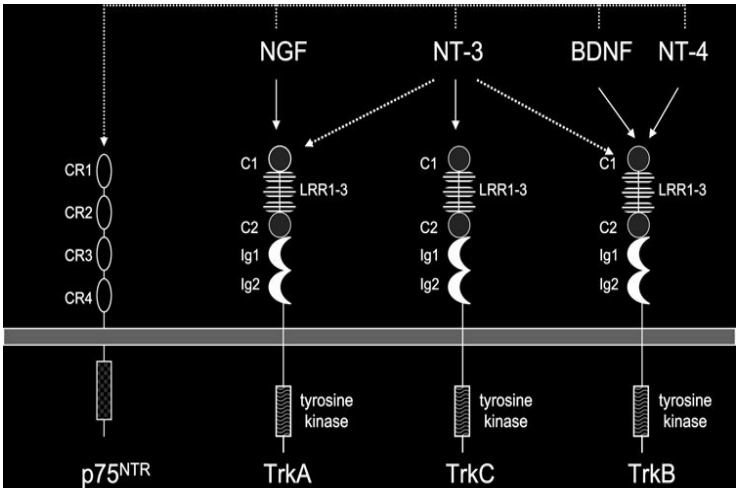


Figure 1.1: Neurotrophins and their corresponding receptors

The family of neurotrophins display selective binding to three Trk receptors: NGF:TrkA, BDNF and NT-4:TrkB, NT-3:TrkC. Image Reference, (Skaper 2012)

For the purpose of this study, any further background will concentrate on NGF/proNGF-mediated signalling pathways and mechanisms. For activation of intrinsic pathways, mature NGF simultaneously engages two TrkA receptors, resulting in receptor dimerisation and trans-phosphorylation (Ibanez et al., 1993). To enhance activation, the widely expressed p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor (TNF) receptor superfamily, collaborates with TrkA to form a high affinity neurotrophin complex (Berg et al., 1991; Hempstead et al., 1991; Esposito et al., 2001; Barker 2004).

While $p75^{NTR}$ can modulate NGF/TrkA trophic signalling (Verdi et al., 1994; Brennan et al., 1999; Barker 2004; Kuruvilla et al., 2004), Trk-independent $p75^{NTR}$ signalling (Majdan et al., 2001) initiates cell death in developing sympathetic neurons. This has also been demonstrated in SCG cultures (Estus et al., 1994; Ham et al., 1995) and *in vivo* (Brennan et al., 1999). The latter study further illustrates that while a $p75^{NTR-/-}$ knockout model rescues cells during development, it does not alter mature sympathetic neuron numbers in adult mice. It was later verified that $p75^{NTR}$ levels decrease in adult neurons (Cowen et al., 2003) suggesting that $p75^{NTR}$ is developmentally regulated, as discussed later (**1.3.1**).

Previous reports indicate that NGF binding to TrkA overrides the $p75^{NTR}$-mediated death signal (Yoon et al., 1998; Brennan et al., 1999; Friedman 2000), however, some evidence proposes that the complex association between NGF and the $p75^{NTR}$/TrkA heterodimer may actually result in either cell survival or cell death, as reviewed by Friedman and Greene (1999); Ceni et al., (2010). Either way, it is clear that the activation of $p75^{NTR}$-dependent signalling is regulated by the associated co-receptor e.g. TrkA and ligand e.g. NGF (Dechant and Barde 2002; Hempstead 2002; Roux and Barker 2002; Teng and Hempstead 2004).

Despite NGF being assigned a primary role as a trophic factor, it was also shown to influence $p75^{NTR}$-mediated cell death during the early development of retinal neurons (Frade et al., 1996), motoneurons (Sedel et al., 1999); and non-neuronal lineages such as oligodendrocytes (Casaccia-Bonnefil et al., 1996) and Schwann cells (Soilu-Hanninen et al., 1999). However, it was later postulated that this could be the work of the NGF precursor, proNGF (Kaplan and Miller 1997; Jansen et al., 2007). Confusion surrounding the converse actions that pro- and mature neurotrophins have on cell

survival originated when Lee et al., (2001) discovered that a mutated form of proNGF, resistant to cleavage, promoted cell death in a rat pheochromocytoma cell line (PC12 cells) and dissociated neonatal SCG neurons. Further studies confirmed the death-promoting activity of proNGF in various cell types during early development in culture, including oligodendrocytes (Beattie et al., 2002), corticospinal neurons (Harrington et al., 2004), photoreceptors (Srinivasan et al., 2004) and basal forebrain cholinergic neurons (BFCN) of the CNS (Volosin et al., 2006).

The death response is initiated by complex formation of proNGF with $p75^{NTR}$ and sortilin, a member of the Vps10p domain receptor family (Nykjaer et al., 2004; Volosin et al., 2006). Sortilin also binds to other proteins such as neurotensin, sphingolipid activator protein (SAP), and lipoprotein lipase (LpL), and has been implicated in the correct intracellular trafficking of these polypeptides to and from the Golgi apparatus (Petersen et al., 1997; Mazella et al., 1998; Munck Petersen et al., 1999; Nielsen et al., 1999; Lefrancois et al., 2003). Not exclusively associated with proNGF, sortilin also interacts with the BDNF pro-domain and mediates efficient intracellular targeting of proBDNF to the regulatory secretory pathway in neurosecretory and neuronal cells (Chen et al., 2005).

However, in order to elicit cell death, it is believed that sortilin interacts with $p75^{NTR}$ at the cell surface (Nykjaer et al., 2004). An associating factor of sortilin, NRH2, has been identified to act as a trafficking switch to prevent lysosomal sortilin degradation and redistribution of sortilin to the membrane to promote $p75^{NTR}$/sortilin complex formation in response to pro-neurotrophins (Kim and Hempstead 2009).

Despite evidence of death-promoting activities, there have also been reports of proNGF (mutated, cleavage resistant forms) acting neurotrophically in neonatal SCG cultures and PC12 cells (Fahnestock et al., 2004; Clewes et al., 2008; Masoudi et al., 2009). While some studies indicate that proNGF-induced neurotrophic signalling is mediated by TrkA and ERK1/2, it was later discovered that siRNA knockdown of TrkA reduced mNGF- but not cleavage-resistant proNGF-dependent neurite outgrowth of postnatal SCG neurons in culture. Furthermore, $p75^{NTR}$ was implicated in the process of proNGF-dependent neurite outgrowth (Howard et al., 2013). It has been suggested that TrkA-dependent neurotrophic effects of proNGF are not due to a direct interaction but require $p75^{NTR}$-mediated endocytosis and cleavage to mature NGF by furins, as demonstrated in HEK293T cells (Boutilier et al., 2008). Peri-somatic

glia have also been implicated in sortilin and $p75^{NTR}$ dependent cleavage of proNGF to mNGF, which subsequently induces neurite outgrowth via TrkA in adult mouse DRG neurons (Kalous et al., 2012). However, in the Howard et al., (2013) study, which uses compartmental cultures, proNGF-induced neurite outgrowth is observed in postnatal mouse SCG neurons, even in the absence of glial support.

Altogether, the availability of pro-protein convertases e.g furin, proteases e.g MMP-7, plasmins, or surrounding glia for cleavage of proNGF may help to determine the final signalling outcome, in addition to the availability of relevant receptors and downstream effectors. Protein binding affinities can also be a governing factor. ProNGF possesses a high affinity for $p75^{NTR}$ (Lee et al., 2001; Nykjaer et al., 2004) and preferentially binds $p75^{NTR}$ over TrkA (Lee et al., 2001), which may explain why selective binding of $p75^{NTR}$, along with the availability of sortilin, leads to apoptotic death of cells that express both TrkA and $p75^{NTR}$.

Another important consideration to make when assessing data is that several proNGF studies have extracted information from diverse cell types, including developing sympathetic, sensory and central neurons, as well as cell lines. Not only are there variations in cell receptor expression across cell types, but changes in physiological functions and growth patterns may also contribute to differing proNGF signalling responses. Furthermore, studies have been conducted with the use of various mutated, cleavage resistant proNGF (**see proNGF section 3.3.1**), as well as wild type proNGF, something that must be taken into account when evaluating research.

1.3.1 Neurotrophic signalling in ageing

While neurodegeneration of ageing sympathetic systems was originally considered to be associated with reduced neurotrophic target support (Cowen et al., 2003), it was later suggested that the unprocessed pro-neurotrophin peptides could facilitate this process (Al-Shawi et al., 2008).

Several studies had previously demonstrated proNGF neurotoxicity during early development or *in vitro*, (Nykjaer et al., 2004; Pedraza et al., 2005; Volosin et al., 2006). However, in 2008, Al-Shawi et al. showed that subpopulations of central and peripheral neurons derived from old rodents were killed by proNGF, and observed a

significant reduction in proNGF-mediated neuronal loss when using Neurotensin, a physiological ligand for sortilin, on aged brain slices (Al-Shawi et al., 2008). This complemented studies that revealed an increase in sympathetic neuron survival in sortilin-deficient aged animals, greater than a year old (Jansen et al., 2007). In the Jansen et al., (2007) study, sortilin expression was shown to increase the affinity of pro-neurotrophins to $p75^{NTR}$ by 100-fold. Furthermore, data illustrated an up-regulation of sortilin in vulnerable populations of basal forebrain cholinergic neurons (BFCN) and SCG neurons in aged rodents (Al-Shawi et al., 2008) and an increase in proNGF levels in the projection areas of some of these populations (Bierl and Isaacson 2007; Al-Shawi et al., 2008). These findings, together, confirmed that proNGF and sortilin play a key role in mediating neurotoxic effects in neurotrophin-responsive neurons in ageing. Further evidence has also demonstrated the neurotoxicity of proNGF in response to injury in adult mice (Harrington et al., 2004) and during AD in human cortical neurons (Peng et al., 2004; Pedraza et al., 2005).

Neurotrophic activities of proNGF have also been observed in adult neurons (Al-Shawi et al., 2008). Although the conditions which determine the ultimate outcome of cell activities through proNGF are still unknown (Ibanez 2002), it is likely that the ratio of TrkA to $p75^{NTR}$/sortilin expressed at the cell surface is a contributing factor (Fahnestock et al., 2004; Al-Shawi et al., 2008; Clewes et al., 2008; Masoudi et al., 2009). Reverse transcription polymerase chain reaction (RT-PCR) on SCG neurons by Cowen et al., (2003) found that TrkA expression remained stable during mature adulthood and old age, while $p75^{NTR}$ expression is largely reduced. However, dynamic regulation of the latter occurs after insult or injury in the brain (Kokaia et al., 1998; Roux et al., 1999; Ramos et al., 2007) and spinal cord injury models (Ernfors et al., 1989; Koliatsos et al., 1991; Hayes et al., 1992). Further studies indicate that this is variable and may depend on the cell type; $p75^{NTR}$ down-regulation in sensory neurons but up-regulation in local glial cells has been observed, post injury (Li et al., 2008).

Altogether, a local up-regulation of $p75^{NTR}$, combined with higher levels of proNGF, released after injury (Beattie et al., 2002; Volosin et al., 2008), supplemented by the presence of sortilin may shift the balance of neuronal fate towards cell-death and may constitute a mechanism to eliminate damaged cells. Jansen et al., (2007) adds that, in SCGs, this is coupled by a reduction of target-derived mature neurotrophins during age-dependent axonal dystrophy.

1.3.2 Neurotrophic signalling - Downstream

NGF engagement of Trk receptors initiates survival-promoting signal transduction in developing sympathetic neurons through activation of GTP binding protein Ras, which mediates two intracellular pathways: Mitogen-activated protein kinase (MAPK)/extracellular signal-related kinases (ERK) (Xia et al., 1995; Nobes et al., 1996; Grewal et al., 1999; Mazzoni et al., 1999; Kaplan and Miller 2000) and phosphatidylinositol 3-kinase (P13K)/AKT (Holgado-Madruga et al., 1997; Philpott et al., 1997; Crowder and Freeman 1998; Vaillant et al., 1999). Both pathways also have a role to play in axon and neurite outgrowth (Markus et al., 2002; Song and Yoo 2011) by maintaining and steering growth cone structure (Song et al., 1997; Ming et al., 2002; Atwal et al., 2003). P13K can also be activated independently of Ras (Datta et al., 1999; Yuan and Yankner 2000) and signals other downstream effectors, which influence survival e.g. inhibitor of apoptosis (IAP) family of caspase inhibitors (Wiese et al., 1999).

The P13K/Akt pathway promotes survival by directly inhibiting the activity of apoptotic proteins, e.g. Bad and Forkhead, or through blocking c-Jun terminal kinase (JNK), members of the MAPK family, and subsequent p53-Bax-dependent apoptosis. It also works by indirectly increasing anti-apoptotic proteins, e.g. IAP and Bcl-2 (Datta et al., 1997; Kaplan and Miller 2000). In contrast, MEK/MAPK promotes NGF-dependent survival by directly stimulating the activity or expression of anti-apoptotic proteins, including Bcl-2 (Aloyz et al., 1998) and the transcription factor CREB (cAMP response element binding protein) (Riccio et al., 1999). NGF was found to significantly increase Bcl-2 levels in sympathetic neurons (Aloyz et al., 1998), which subsequently protected these and other neurons from apoptotic cell death (Michaelidis et al., 1996). Further detail into the pathways controlled through these proteins has been reviewed (Kaplan and Miller 2000; Huang and Reichardt 2003).

Rac1 and Cdc42, members of the Rho GTPase family, are also implicated in NGF-dependent signalling and have been shown to positively regulate neurite extension through re-organisation of the actin cytoskeleton, via a $p75^{NTR}$-dependent pathway (Yamashita et al., 1999; Harrington et al., 2002). Both Rac1 and Cdc42 are activated very early in response to NGF in PC12 cells (Aoki et al., 2004). Immediately after NGF addition, Rac1 and Cdc42 are transiently activated in the periphery and recurrent activation is also observed in the tips of the protrusions.

Following $p75^{NTR}$ activation, the recruitment of adaptor protein complexes is necessary to promote further signalling due to a lack of intrinsic catalytic activity (Hempstead 2002; Bandtlow and Dechant 2004; Gentry et al., 2004; Hasegawa et al., 2004). Several of these have been associated with trophic deprivation-dependent apoptosis in developing sympathetic neurons. These include NRIF (Casademunt et al., 1999; Linggi et al., 2005), TRAF6 (Yeiser et al., 2004), and the MAGE family members: NRAGE (neurotrophin receptor–interacting MAGE homologue) (Salehi et al., 2000; Kendall et al., 2002; Ceni et al., 2010) and Necdin (Tcherpakov et al., 2002; Kuwako et al., 2004).

The $p75^{NTR}$ adaptor protein, NRAGE was implicated in $p75^{NTR}$-dependent cell death of NGF-dependent sympathetic neurons during development, through activation of JNK (Salehi et al., 2000; Kendall et al., 2002). Not solely associated with NGF signalling, sympathetic neurons derived from a neonatal NRAGE knockout mouse model were also found to be resistant to BDNF/$p75^{NTR}$/JNK-mediated cell death (Bertrand et al., 2008). However, less is known about the impact of NRAGE and other adaptor proteins in NGF-responsive neurons during ageing.

Rac1 and Cdc42, MAPK activators, are also involved in JNK-dependent apoptosis, following NGF withdrawal, in developing sympathetic neurons. Over-expression of constitutively active forms of Rac1 and Cdc42 leads to JNK activation, and results in the subsequent death of sympathetic neurons. Conversely, overexpression of dominant-negative mutants of Cdc42 and Rac1 in sympathetic neurons prevents elevation of JNK, c-Jun transcription factor, and cell death (Bazenet et al., 1998).

1.3.3 JNK responses

JNKs have received considerable attention since their initial discovery 20 years ago (Kyriakis and Avruch 2001). Essential for the regulation of growth and survival signalling, they play an important role in the formation of the developing nervous system, but also in response to injury or insult of the adult nervous system (Yamasaki et al., 2012) and age-related diseases (Brecht et al., 2005).

The significance of JNK signalling in $p75^{NTR}$-mediated apoptosis, following trophic withdrawal in early development, has already been established in sympathetic neurons (Eilers et al., 1998; Eilers et al., 2001), central neurons (Friedman 2000; Troy et

al., 2002; Bhakar et al., 2003; Becker et al., 2004) and non-neuronal cells (Casaccia-Bonnefil et al., 1996; Beattie et al., 2002).

In ageing, the focal point of JNK research has remained with neurodegenerative diseases; in the CNS, e.g. Parkinson's disease (PD) (Brecht et al., 2005), Alzheimer's disease (AD) (Morishima et al., 2001; Zhu et al., 2001; Savage et al., 2002; Vogel et al., 2009; Killick et al., 2014), and in the PNS, e.g. myelin breakdown (Gomez-Sanchez et al., 2015). These associations have led to the investigation of JNKs as potential therapeutic targets for neuroprotection (Manning and Davis 2003; Bogoyevitch et al., 2010; Antoniou et al., 2011). *In vivo* studies on adult sensory neurons have also reported JNK involvement in the process of regeneration (Kenney and Kocsis 1998; Barnat et al., 2010; Ruff et al., 2012), which is discussed later (**Results 3.1.1.4**).

It is believed that the multi-faceted nature of JNK regulation in the nervous system leads to an integrated and input-balanced signalling outcome (Morrison and Davis 2003) whereby differential regulation can be determined by the age of the neuron or the state of the environment. Different cell types have varying outcomes as well e.g. while survival of neonatal sensory neurons is reduced by JNK inhibition in the presence of NGF, there is no effect on the survival of cultured sympathetic neurons from the same developmental stage (Lindwall and Kanje 2005). Similarly, neurite outgrowth is JNK-dependent for early postnatal hippocampal but not for cortical neurons (Eminel et al., 2008).

JNK signal variation can be applied through different isoforms. The JNK family consists of three related genes: JNK1 and JNK2, which are present in most tissues, and JNK3, which is predominantly found in the brain and testes. Diversity is accomplished by splicing each gene to create ten JNK isoforms, all of which share an epitope that requires dual phosphorylation for JNK activation. Located downstream of various signalling pathways, activated phospho-JNKs subsequently phosphorylate several protein substrates (~50 have been discovered). JNK substrates are localised throughout the cell including dendrites, axons and presynaptic endings (Coffey et al., 2000; Chang et al., 2003; Bjorkblom et al., 2005; Tararuk et al., 2006). Proteins residing in the nucleus that are phosphorylated by JNK include transcription factors (AP-1, c-Jun, ATF2 and p53) and hormone receptors, while non-nuclear substrates include those involved in protein degradation (the E3 ligase Itch), signal transduction (adaptor and scaffold proteins, and other kinases), apoptotic cell death (mitochondrial

Bcl-2 family members), and cell movement (paxillin, doublecortin (DCX), microtubule associated proteins (MAPs), and stathmin (SCG10) (Hibi et al., 1993; Kawauchi et al., 2003; Gdalyahu et al., 2004; Bogoyevitch and Kobe 2006; Tararuk et al., 2006).

Stress-induced or physiological responses facilitate MKK4 and MKK7 phosphorylation and activation of JNK via a sequential MAPK cascade (**Fig 1.2**) (Wang et al., 2007; Asaoka and Nishina 2010; Haeusgen et al., 2011). Binding of MKK4 and MKK7 to D domains in the N-terminal region of JNKs is very specific e.g. JNK1 and JNK2 do not interact with the same domains of other MKKs (Bardwell et al., 2009). Whilst MKK7 has single specificity for JNKs, MKK4 can also phosphorylate p38 MAPKs (Davis 2000), which must be taken into account when targeting MKK4. The binding affinity of each isoform to MKK is also different, and can influence signal diversity i.e. MKK4 has a higher binding affinity to JNK1 and JNK3 than JNK2 (Ho et al., 2003).

Signal differentiation may also be achieved by intracellular concentrations of binding partners, which can determine the resulting inhibitory or activating effect of MKKs on JNKs (Kieran et al., 1999). Following JNK activation, the formation of multi-enzyme complexes, which include the scaffold JNK-interacting proteins JIP1 to JIP4 (Dickens et al., 1997; Whitmarsh et al., 1998; Ito et al., 1999; Yasuda et al., 1999; Kelkar et al., 2000), and other proteins, drives regulation and contributes to signal specificity by binding and sequestering selective MAPK components to a restricted region of the cell, depending on the type of stimulus.

Therefore, localisation of JNK can also be a defining factor; nuclear JNK influences JNK-mediated cell death after the withdrawal of neurotrophic support in neonatal CNS neurons (Bjorkblom et al., 2008), while effects on neurite outgrowth can be mediated by JNK phosphorylation of cytoplasmic targets, as displayed in the developing cerebellum (Bjorkblom et al., 2005), cortex (Westerlund et al., 2011) and also during axonal regeneration in adult DRG (Barnat et al., 2010). It is important to note that when observing subcellular compartmentalisation, JNKs are not always static e.g. JNK3 translocates from the cytoplasm to the nucleus in pyramidal neurons of the hippocampus, in response to stress induced by hypoxia (Zhang et al., 1998).

The outcome of JNK signalling can also be influenced by the time course of activation. Sustained JNK activation produces an apoptotic response in cerebellar granule

neurons (CGN) (Chen and Tan 2000) whereas, in human breast carcinoma cells, many cytokines cause only transient JNK activation, making apoptosis less likely (Liu et al., 1996). Cells may also interpret transient JNK activation as a survival signal because of the activation state of other signalling pathways within the cell. It has been previously described that during most forms of environmental stress, apoptosis is inactive because the JNK-dependent apoptotic signalling pathway is blocked by activation of survival signalling pathways (Xia et al., 1995) such as Akt/P13K, and ERK.

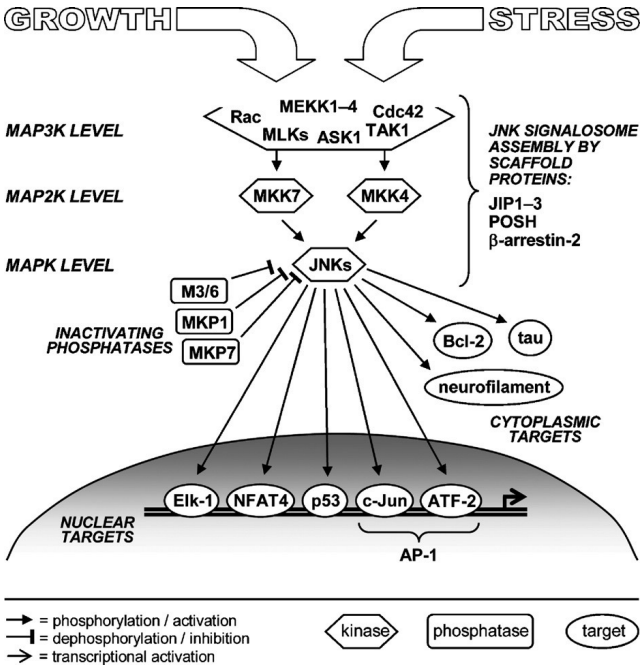


Figure 1.2: JNK cascade model in neuronal growth and stress signalling

The three-tiered core signalling module of MAPK, MAPKK and MAPKKK (signalosome) is composed by scaffold proteins in a situation- and cell type-specific manner (Waetzig et al., 2006).

The manner by which JNK isoforms operate can facilitate different outcomes as well. In a kainic-acid induced injury (excitotoxicity) model in mouse hippocampal neurons, JNK1 knockouts increased site-specific neurodegeneration, while seizures were

prevented in JNK3 KOs (Brecht et al., 2005). This is supported by post-injury ischemic (transient middle cerebral artery occlusion) *in vivo* studies (stroke model), in rat brain mitochondra, where the number of MKK4:JNK3 complexes rises but the concentration of MKK4:JNK1 decreases (Zhao and Herdegen 2009).

Although JNKs are mainly known for their function in pro-apoptotic signalling, their influence on neurite outgrowth in development (Coffey et al., 2000; Oliva et al., 2006; Tararuk et al., 2006; Qu et al., 2013) and in the regeneration of the damaged adult peripheral nervous system (Kenney and Kocsis 1998; Lindwall et al., 2004; Barnat et al., 2010; Ruff et al., 2012) has also been documented. Isoform-specific activation has also been observed here; in the PNS, neuritogenesis is delayed by lack of JNK2 and JNK3, but not JNK1 during the axonal regeneration of adult mouse DRG. However, sustained neurite elongation relies on JNK1 and JNK2 (Barnat et al., 2010).

As mentioned, age is also a defining factor in the influence of different isoforms e.g. JNK1 and JNK2 are important for regulating apoptosis in the developing nervous system (Kuan et al., 1999), while JNK3 is a key mediator in $p75^{NTR}$-dependent apoptosis of neonatal neurons following trophic deprivation (Bruckner et al., 2001; Pirianov et al., 2007; Kenchappa et al., 2010) but also during the neurotoxicity associated with ageing and disease (Brecht et al., 2005). Further JNK knockout phenotypes have been reviewed (Bogoyevitch and Kobe 2006; Coffey 2014).

While research has centred around isoform-specific JNK inhibition as a way to confer neuroprotection and advance our understanding of signalling mechanisms associated with neurodegenerative diseases in the brain, there have been fewer studies on the effects of JNK inhibition in the ageing PNS.

1.4 Peripheral Nerve Injury

Peripheral nerves are less protected than the CNS, which utilises the blood–brain barrier (BBB) to defend against penetration of proteins and other molecules from the circulating blood. This leaves the PNS neurons exposed to toxins and mechanical injuries and is perhaps why peripheral networks have evolved the ability to regenerate.

Axon injury can be caused by trauma, toxic insults, and neurodegenerative and genetic disorders (Coleman 2005; Wang et al., 2012), resulting in a loss of motor, sensory or autonomic control of target organs. Enforced changes can be observed within the external cell environment, including nutrients, growth factors, cytokines and cell matrix adhesion factors (Ide 1996). In the CNS, injured axons struggle to regenerate due to a combination of low intrinsic growth potential and environmental factors (Bradke et al., 2012), while injured neurons in the PNS can show robust regeneration. In many cases, this renewal is slow and the target cell degenerates or becomes non-functional before re-innervation eventually occurs (Bradke et al., 2012). This can be hindered even more in ageing (Tanaka and Webster 1991; Verdu et al., 2000; Pan et al., 2003; Kennedy and Zochodne 2005), primarily due to decreased macrophage recruitment and Schwann cell (non-neuronal) reactivity (Komiyama and Suzuki 1992).

The process of regeneration is triggered, following injury, when local responses promote resealing of the injured axon and re-organisation of the cytoskeleton to make way for growth cone formation (Bradke et al., 2012). Concurrently, local signalling pathways activate and promote retrograde transport of injury signals to the nucleus, which subsequently initiates a pro-regenerative transcriptional program (Tedeschi 2011) and shifts neurons into a regenerative state. Efficient axon regeneration is delayed until this state has been induced (Smith and Skene 1997).

More specifically, the affected area undergoes a four-step regeneration process, which includes Wallerian degeneration (breakdown of the separated axon) and chromatolysis (dissolution of Nissl bodies within neurons), axonal regeneration, target innervation and axonal maturation (Verdu 1998). At 2-3 days after injury, in order to remove any unwanted debris, there is an influx of macrophages within the degenerating nerve. To assist with this process, macrophages secrete cytokines to

promote the mitosis of reactive denervated non-neuronal Schwann cells, which become phagocytic. Schwann cells help with the degradation of myelin debris, but also secrete neurotrophic factors to promote axon elongation.

In adult and aged sympathetic networks, a number of studies have demonstrated that NGF can promote regeneration and increase the sympathetic innervation of peripheral targets in injured (Bjerre et al., 1975; Saffran et al., 1989) and uninjured axons (Isaacson et al., 1990; Saffran and Crutcher 1990; Isaacson and Crutcher 1998). The latter studies, performed *in vivo*, reported that intra-cerebral NGF administration induces sympathetic hyper-innervation of target CV vessels in aged rat. Administration using miniosmotic pumps (Andrews and Cowen 1994a) also demonstrated that exogenous NGF could induce organotypic neurite outgrowth from aged neurons undergoing nerve fibre atrophy. Here, dendritic arborisation (dendritic and cell body growth) was observed in response to NGF but primary dendrites and branch points were not affected. While they are still NGF-responsive, aged SCG neurons exhibit a decreased ability to mediate collateral sprouting in response to a partial denervation of a target (Kuchel and Zigmond 1991; Kuchel 1993) or following the exogenous administration of physiological levels of NGF (Gavazzi 1995). Interestingly, Andrews and Cowen (1994a) revealed that administration of NGF into the middle cerebral artery of aged rats, induced greater nerve growth in aged compared to young nerves, suggesting differential and developmental regulation.

A commonly used method for studying the effects of injury on the nervous system is the sectioning of nerves (axotomy), usually postganglionic, which causes a variety of pathological changes in the neuronal somata (Taxi and Eugene 1995). Not long after axotomy, thin axonal sprouts or growth cones develop from the proximal nerve segment, which create amoeboid-like extensions to allow elongation of nerves. Growth is the result of increased synthesis in the neuronal body of proteins and other materials that pass distally to the axon tip through axonal transport. The direction of the advancing tip changes depending on the physical and chemical properties of the adjacent distal environment and the neurotrophic factors produced by non-neuronal cells (Fawcett and Keynes 1990).

After sprouting has been initiated, Schwann cells secrete components of basal lamina to stimulate and guide regeneration. The relevant molecules include laminin, fibronectin and several others embedded within the extracellular matrix (ECM), the environment that provides structural and biochemical support to the surrounding

cells. The regenerating axons express integrins to mediate recognition of the ECM and the subsequent intracellular signalling that facilitates neuronal growth. Del Signore et al., (2006) identified changes in the expression of a number of molecules in the rat SCG after postganglionic axotomy including collagen α1 type I and III, intermediate filament proteins, and modulators for neurite outgrowth such as thrombin receptor and BMP-4. Once muscles are re-innervated, maturation completes the process and restores nerve conduction through myelination, although this is delayed in ageing. Over 200 genes in adult rat SCG were found to have alterations after axotomy (Boeshore et al., 2004), making it an exceedingly complex topic. As discussed before, $p75^{NTR}$ up-regulation is also observed in Schwann cells (Lemke and Chao 1988; Taniuchi et al., 1988) and oligodendrocytes (Beattie et al., 2002) following peripheral damage and has been shown to be important for Schwann cell myelination and re-myelination during regeneration (Cosgaya et al., 2002).

Post-injury repair in the periphery has traditionally been researched *in vivo*, either by transplantation studies (Gavazzi et al., 1992; Gavazzi and Cowen 1993a; Gavazzi and Cowen 1993b), conditioning lesion or drug application into the DRG, or sciatic nerve, followed by an axonal growth assay, either *in vivo* or in culture (McQuarrie and Grafstein 1973; Kilmer and Carlsen 1984; Smith and Skene 1997; Neumann et al., 2002; Qiu et al., 2002). At the time of this study, it was recognised that axotomy of peripheral neurons (DRG) by dissociating and then culturing them also initiates the activation of injury-activated transcription factors and up-regulation of regeneration-associated genes (Zou et al., 2009; Saijilafu et al., 2013). Furthermore, this injury activates neurite outgrowth in a manner similar to *in vivo* conditioning lesions. Although this has been observed in sensory neurons, the same may also be true for sympathetic motor neurons, and culturing these neurons could provide an insight into the processes that are associated with injury *in vivo*.

1.5 Glia

Historically, glial cells have been treated as passive non-excitable elements of the nervous system, mainly providing neuronal support. Pre-assigned roles include maintaining a functional synaptic environment to guiding developing axons, forming the blood-brain barrier, nourishing neurons, and myelinating axons. However, over

the last two decades, evidence has gathered to challenge this traditional view. The use of animal models has shown that actually there is not a single function in the nervous system that occurs without the contribution of glial cells. Therefore, for neuroscientific progression, further understanding of the interaction between neurons and glia is absolutely vital.

The majority of neuronal synapses are enveloped by, or in contact with glial cell processes (Auld and Robitaille 2003; Hanani 2010). In the CNS, there are three types of glial cell: astrocytes, oligodendrocytes and microglia, while satellite glial cells and Schwann cells reside in the PNS. As discussed before **(1.4),** Schwann cells have an important role during peripheral nerve regeneration, however, another role for these cells is to confer neuroprotection and axon protection, a property shared by astrocytes in the brain. Similarly, there is evidence to indicate that satellite glial cells have a significant role in the response to nerve damage (Hanani 2010).

Glial cells also release trophic factors, neuroprotective molecules, and neurotransmitters essential for neuronal survival and function (Toku et al., 1998; Tanaka et al., 1999; Hansson and Ronnback 2003; Montana et al., 2006). Equally, all glia present a variety of receptors for neurotransmitters, hormones and other bioactive molecules (Jabs et al., 2005) thus providing a way for glia and neurons to communicate. Not only do neurotrophins support neurons but they also contribute to glial cell development and survival (Althaus and Richter-Landsberg 2000; Syroid et al., 2000; Chan et al., 2004; Husson et al., 2005; Yamauchi et al., 2005). Like glia in the brain, Schwann cells express receptors for neurotrophins and respond to concentration changes of neurotrophins within the extracellular space. This has been reviewed (Elkabes et al., 1996; Althaus and Richter-Landsberg 2000; Hempstead and Salzer 2002; Chan et al., 2004). In fact, in transected nerves, Schwann cells can survive even in the absence of axons, by establishing an autocrine circuit of trophic factor production (Meier et al., 1999). In degenerative conditions, this endurance of Schwann cells allows them to regulate the survival and differentiation of neurons.

Further downstream, another property that glial cells share with neurons is the regulation of survival and differentiation by JNKs, both in the CNS (Casaccia-Bonnefil et al., 1996; Ladiwala et al., 1998; Yoon et al., 1998; Jurewicz et al., 2003; Pirianov et al., 2006) and the PNS (Parkinson et al., 2004). This is discussed further (**Results 3.2.1**).

1.6 Experimental models in ageing

Research into the process of ageing carries unique challenges and to avoid using invasive methods to study the pathophysiology of neurodegenerative diseases in humans, a variety of animal models have been developed.

Non-surgical models aim to replicate disease conditions that are associated with ageing, and the use of laboratory animals (mainly rodents**)** offers a way to carefully characterise anatomical, physiological and behavioural aspects aligned with these conditions. Recent work in genetics and in comparative biology confirms that ageing exhibits common characteristics across species. It is now apparent that some of the hormones and cellular pathways that influence the rate of ageing in lower organisms also contribute to many of the manifestations of ageing that we see in humans (Sinclair and Guarente 2006).

Although ageing models are expensive and translation to the clinic is not always straightforward, these models have enhanced our understanding of the cellular and molecular mechanisms involved in later life, as well as identifying candidate compounds for use in clinical trials.

Where possible it is important to complement non-surgical studies with *in vitro* studies of wild-type aged models to further define molecular pathways. In particular, genetic manipulations and the use of targeted inhibitory compounds on cultured cells have helped with the molecular dissection of important biological pathways in normal and diseased states.

In the past, the difficulty of maintaining cells derived from adults steered experimental work towards the use of embryonic or postnatal cells, until the first studies in ageing sympathetic neurons began with Uchida and Tomonaga (1985). Dissociated sympathetic neurons from young and aged mice were cultured on different substrata, using either collagen or polyornithine-binding components of heart-cell-conditioned medium (HCM). They found that the ability of sympathetic neurons to respond to HCM is affected in old age, and changes depending on the substratum but also on the parameter measured.

Soon after, different components of the extracellular matrix (ECM) were found to provide spatial cues to growing neurons in development and regeneration *in vivo* (Sanes 1989; Reichardt and Tomaselli 1991; Letourneau et al., 1994). Many experimental studies then recognised the importance of protein constituents in the (ECM) in influencing some aspects of neural differentiation. For example, Cowen et al., (1997) discovered a positive growth response of aged neurons in the presence of basement membrane glycoprotein, laminin, and NGF. Explant cultures of sympathetic neurons from mature and aged rat SCG were grown on substrata containing different doses of laminin. Only when exogenous NGF was added, was there a powerful effect on nerve growth (Cowen et al., 1997). Collagen type I and collagen type IV were also shown to promote process outgrowth in neurons and non-neuronal cell types (Reichardt and Tomaselli 1991; Letourneau et al., 1994; Cowen et al., 1997) in the presence of NGF (**see Materials & Methods 2.2**).

After the initial work by Uchida and Tomonaga (1985), further studies failed to pinpoint the possible contributions of serum and non-neuronal cells to the survival and growth of neurons in culture. So, Orike et al., (2001a) established a technique for the dissociation, purification and culture of sympathetic neurons from adult and aged rat SCG under serum free conditions, and in defined media. Crucially, this method produced a high yield of viable growing neurons, meaning that subpopulations of neurons could be sampled, and gave a better indication into the influence of surrounding factors.

(Orike et al., 2001a) describes the viability of neurons in culture as being underpinned by two vital steps:

(i) enzyme digestion, which is adjusted depending on the age of the donor animal. Extended incubation times with collagenase are important for the successful dissociation of adult and aged neurons.

(ii) sequential trituration, with a fire-polished pipette, the tip diameter of which is close to the size of the segments of ganglion being dissociated. The process of trituration (**see Materials & Methods 2.2**) generates approximately 10% of the total number of neurons from aged ganglia, 30% from neonatal ganglia and an intermediate value for young adult ganglia.

The use of small-interfering RNAs (siRNAs) in animal cultures has provided another useful way to study protein function in neurons using a highly efficient and non-toxic method. First discovered by Fire et al., (1998), RNA interference is a process by which double stranded RNAs (siRNAs), once delivered inside a cell, are processed into short RNA duplexes, which have the ability to promote mRNA degradation and the silencing of specific genes (Felekkis and Deltas 2006). Synthetic siRNAs linked to cell-penetrating peptide Penetratin-1 has allowed for quick and effective uptake of siRNA in whole populations of primary SCG neurons (Davidson et al., 2004).

1.7 Treatment of ageing

Despite being a complex area of research, a growing body of evidence has alerted researchers to the possibility of scientific intervention methods to slow ageing and significantly reduce the risk of age-related disease. Several studies on animal models have demonstrated that ageing rates and life expectancy can be modified, through altering reproduction, reducing caloric intake, or changing the signalling pathways of specific physiological mechanisms (Yu et al., 1985; Brown-Borg et al., 1996; Weindruch and Sohal 1997; Flurkey et al., 2001; Tatar et al., 2003). In fact, drugs to slow the ageing process already exist. Everolimus, a cancer drug, and Metformin, used in Type II diabetes patients, are just two examples of compounds already in circulation that combat specific diseases but possess the ability to slow ageing as well (Anisimov et al., 2008; Harrison et al., 2009; Spong and Bartke 2012; Anisimov 2013b; Anisimov 2013a; Blagosklonny 2013).

However, the administration of a therapy involving proteins in the brain has inherent problems. Because of the blood–brain barrier, the protein must be infused directly, produced by viral constructs, secreted from implanted protein-secreting cells or actively transported across the brain. An alternative to this is to use small molecule agonists, modulators or enhancers to target the receptors or intracellular signalling molecules associated with a specific pathway.

The use of JNK inhibitors (Scapin et al., 2003) has intensified as a neuroprotective strategy (Borsello et al., 2003; Hunot et al., 2004), but also to help deduce the physiological and pathological functions of JNKs, their isoforms, and the molecular pathways that control this. This is discussed in more detail later (**3.1.1.6**).

1.8 Aims of the project

There are marked differences in the survival characteristics between neurons in development compared to those in aged adult animals. While neonatal sympathetic neurons are extremely dependent on NGF for survival, this dependency is largely reduced in ageing (Orike et al., 2001b; Al-Shawi et al., 2008). Despite this, aged neurons are still NGF-responsive and NGF-dependent neurite outgrowth is still viable. The NGF precursor, proNGF, can also promote neuritogenesis of young adult and aged neurons, but has additionally been found to mediate neurotoxicity in a sub-population of NGF-responsive neurons of the aged PNS and CNS.

Differential and developmental regulation of neurotrophic signalling mechanisms can be determined by a number of factors from receptor ($p75^{NTR}$, TrkA, sortilin) expression to the availability of ligands and regulatory proteins within the cell. A large body of work has revealed much about receptors and upstream effectors in ageing but a closer look into downstream effectors is necessary.

The hypothesis in this study is that JNK signalling is important for the regulation of NGF-promoting growth and proNGF-mediated signalling in NGF-responsive young adult and aged peripheral neurons. It is also proposed that NRAGE is involved in this process.

JNK inhibitor studies and siRNA knockdown of NRAGE within a modified cell culture method for adult and aged SCG neurons, which monitors the growth and survival, will seek to determine the impact of JNK and NRAGE on NGF and proNGF signalling. Isoform–specific interactions will also be taken into account and further inhibitor studies will look to verify the importance of different JNK isoforms in neurotrophic signalling within adult and aged sympathetic neurons.

While neurons have been thoroughly studied, glia are also essential to the outcome of various signalling pathways. So, it will also be important to visualise the effects of various inhibitors on SCG glia in culture.

Chapter 2

- MATERIALS & METHODS -

2.1 Animals

Healthy, C57BL/6J x CBA/Ca young adult (3-6 months) and old (24-36 months) male mice were used in all culture experiments from colonies maintained at University College London. Mice were fed RM1 special diet services (SDS) ad libitum (AL). RM1 is composed of 14.4% crude protein, 2.7% oil, 4.6% fibre and 90% dry matter, with a caloric content of 14.7 MJ/kg.

All animal studies were ethically reviewed and approved by the UCL Royal Free Campus Ethics and Welfare Committee and the UK Home Office, and were carried out in accordance with European Directive 86/609/EEC. Mice were killed using carbon dioxide (CO_2) inhalation.

2.2 Young Adult and Aged SCG neuron Cell culture

Dissociated primary adult SCG neuron cultures were prepared as previously described (Gavazzi et al., 1999; Orike et al., 2001b; Al-Shawi et al., 2008). Briefly, 3-4 SCGs were aseptically excised and desheathed in Hanks medium containing Hepes buffer (0.01M) and Penicillin/Streptomycin (0.05mg/ml), then placed on ice. Exposed SCGs were cut into 4-5 pieces then digested with 2ml Hanks containing collagenase type XI (1mg/ml) (Sigma, UK) and bovine serum albumin (BSA) (6mg/ml) (Sigma, UK) for approximately 30 mins to 1 hour, at 37°C and 5% CO_2, in a humidified incubator. SCGs were then washed in Hanks solution twice before the addition of 2ml Hanks containing Trypsin (1mg/ml) (Sigma) and BSA (6mg/ml), for ten minutes at 37°C and 5% CO_2, in a humidified incubator. Ganglia were washed twice in Hanks medium and pelleted by centrifugation (1min x $42g_{max}$) in between. Ganglia were mechanically dissociated in 500µl of Neurobasal-A medium (NBM) supplemented with B27 (Gibco, UK), L-Glutamine 2mM and Penicillin/Streptomycin (0.05mg/ml) and 3µM cytosine arabinofuranoside (AraC; Sigma, UK) for 24 hours, unless otherwise indicated. For proNGF cultures (**Results 3.3**), some experiments were carried out with B27 supplement in the absence of insulin (Gibco, UK). Trituration was performed using sterilised, siliconised, fire-polished glass pipettes firstly with a pore size of 0.30-0.40mm and then subsequently with a pore size of 0.15-0.20mm. A maximum of 4 triturations were carried out before supernatant medium was decanted into another Falcon tube. Fresh medium (approx 300-500µL) was added to the original Falcon tube and a further 4 triturations were performed before

the supernatant was decanted again. This process was repeated until no cell clusters could be seen and the majority of neurons were removed. Neurons were plated on glass coverslips (VWR International) at a density of 300/well unless otherwise stated. Pre-sterilised* coverslips were pre-coated by sequential incubation with human collagen IV (Sigma) for 5 hours at 37°C and 5% CO_2, then at 4°C until use. Once plated, and after the inclusion of various factors, cultures were allowed to settle for 20 minutes before incubation at 37°C in a humidified 5% CO_2 chamber.

Neuronal survival and neurite outgrowth was followed for 24 hours of culture, unless otherwise stated. When obtaining data for 72 hours, similar survival and growth rates relative to controls were acquired for consecutive days.

*Coverslips were placed in a glass petri dish containing acetone for 40 minutes then absolute ethanol for 20 minutes at room temperature. Ethanol was decanted followed by the addition of 0.1M Hydrogen Chloride in the fume cupboard. Coverslips were rinsed 3 times in 18.2 MΩ·cm sterile H_2O followed by 8 washes x 3 minutes. 3 more rinses were performed before placing in the drying cabinet. The petri dish was mounted on a shaker during washes to allow complete coating of the coverslips.

Table 2.1: Growth Factors

Factor	Source
NGF (murine 2.5S)	Promega, UK
Furin cleavage-resistant proNGF (mutated-mouse) N-255	Alomone labs
Human recombinant proNGF	Scil Proteins Axxora

Robust growth of rat SCG neurons was observed previously in adult neurons cultures when NGF (Promega mNGF 26kDa) and proNGF were used at 0.4nM (Orike et al., 2001a; Al-Shawi et al., 2008), similar to the physiological concentrations measured in adult rat SCG and iris (Cowen et al., 1996). Therefore the same concentration was used in this study unless otherwise stated.

2.2.1 JNK inhibitors

Table 2.2: JNK inhibitors

All inhibitors were purchased from Calbiochem, U.K.

Inhibitor	Mode of Action
AEG3482 (6-Phenylimidazo[2,1-b]-1,3,4-thiadiazole-2-sulfonamide)	Binds HSP90 and disrupts the association with heat shock protein transcription factor (HSF-1). Freely available HSF-1 subsequently induces the expression of HSP70, which blocks JNK activation
JNK Inhibitor II Also known as SP600125 SAPK Inhibitor II (anthra[1,9-cd]pyrazol-6(2H)-one)	Cell-permeable peptide and reversible, ATP competitive inhibitor. Exhibits over 300-fold greater selectivity for JNK than ERK1 and p38-2 MAP kinases. Inhibits phosphorylation of *c*-Jun. IC50: Jnk1/2=40nM Jnk 3=90nM
JNK Inhibitor III (Ac- YGRKKRRQRRR-gaba-ILKQSMTLNLADPVGSLKPHLRAKN-NH2)	Cell-permeable peptide. Specifically disrupts c-Jun/JNK complex formation and the subsequent phosphorylation and activation of c-Jun by JNK both *in vitro* and intact cells. Can complement JNK signalling studies with JNK Inhibitor II, since its mode of inhibition is different. *Purity: ≥95% by HPLC.*
JNK Inhibitor VIII (N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl) acetamide	Cell-permeable pyridinylamide compound. ATP-competitive, reversible inhibitor of JNK 1 and 2, and JNK 3 at a higher concentration. Displays selectivity over 72 other kinases. Inhibits c-jun phosphorylation with an EC of 920 nM in HepG2 cells. K_i = 2 nM, 4 nM and 52 nM for JNK1, 2 and 3, respectively
JNK Inhibitor IX N-(3-Cyano-4,5,6,7- tetrahydro-1-benzothien-2-yl)-1-naphthamide	Potent ATP-binding site target of JNK 2/3. Under the assay conditions. JNK2 and JNK3 (pIC50 = 6.5 and 6.7) with selectivity over JNK1, p38 α and a panel of > 30 other kinases (pIC_{50} = < 5.0). *Purity: ≥95% by HPLC.*

Previously published information about JNK inhibitor potency in cell-based assays helped to determine the administered concentration in the culture medium of 10μM, at the time of plating (Bennett et al., 2001; Holzberg et al., 2003; Salehi et al., 2006; Szczepankiewicz et al., 2006; Angell et al., 2007; Vivanco et al., 2007; Gao et al., 2013). A higher concentration is necessary with competitive inhibitors (e.g. **JNK inhibitors II**, **VIII** and **IX**) due to a high ATP concentration within cells and/or active efflux of these compounds in neurons/glia in cell-based experiments, compared with biochemical assays on purified proteins.

JNK inhibitor III, a pan-JNK inhibitor that disrupts the JNK-c-Jun interaction (**Table 2.2**), was used at a 10-fold lower concentration than previously reported (Holzberg et al., 2003) to avoid non-specific effects.

2.2.2 Counting

Survival and growth was monitored using a Nikon microscope to count phase bright neurons at 10x magnification. Neurons were counted by myself and one other person (Ivana Slamova), which was consistent across all experiments. This quickened process helped to limit exposure of the cell culture plates to unfamiliar and potentially harmful culture conditions. It also allowed for triplicates of each condition per experiment. Throughout the study, each experiment had plating count of (n=300) unless otherwise stated. Plates were scored at 24 hour intervals. Due to the inherent variability in the survival of untreated control neurons isolated from aged mice, results from representative single experiments are shown as these are internally consistent. Experiments were repeated in triplicate, and results were only deemed to be significant if there was concordance between repeats.

For analysis of survival, neurons were marked as alive or dead and, for analysis of growth, live neurons were classified as growing or non-growing. Live neurons were identified by their large phase-bright, intact cell bodies and neurites (Orike et al., 2001b), and were clearly distinct from dead neurons, which were dark, possessed fragmented soma and neurites, and were often found partly detached from the substrate. Glia were characteristically small and had spindle-shaped phase-grey cell bodies. The following counting criteria were adhered to over the course of the study:

Survival was determined by the number of neurons observed as a percentage of the total number of neurons in the well at time of plating: Criteria were as follows:

- Phase-bright attached neurons were counted. Detached floating neurons were not counted
- Phase-bright round neurons without neurites were counted
- Glia were not counted
- Neurons attached to glia were not counted **(Fig. 2.1; A+B)**
- Neurons with touching cell bodies were not counted

Neurite outgrowth was determined by the number of neurons detected as growing as a percentage of the total number of living neurons, on that particular day. Neuronal growth was marked if any one of the neurite extensions was longer than the diameter of the cell body **(Fig. 2.1; C+D)**.

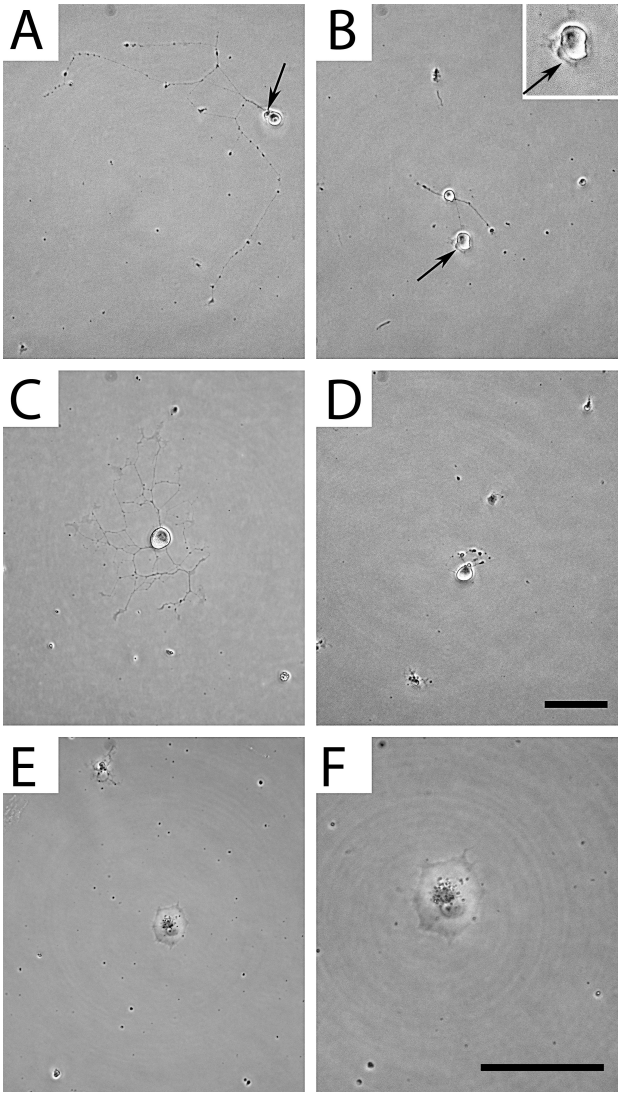


Figure 2.1: Phase contrast images of counting criteria

Scale bar (D) represents 100µm in panels A-E. Scale bar (F) represents 100µm in that specific panel . (A) and (B) show glia attached to neuronal cell bodies, and would not be counted. (C) a typical growing neuron. (D) a neuron projecting neurites that were not longer than the diameter of the cell body and so would be marked for survival, but not growth. (E) a dead neuron (F) the same neuron as E at double the magnification.

2.2.3 Hoechst Assay

Four day old primary cultures on glass coverslips were fixed for 20 minutes with 4% paraformaldehyde (PFA). Coverslips were removed using forceps and placed in coverslip carriers prior to being dipped and washed in PBS three times. Primary antibody was then applied for two hours at room temperature (RT). Following a series of washes in phosphate buffered saline (PBS), the secondary antibody was added for 45 minutes (RT). After the last series of washes, stained cultures were mounted on microscope slides with Fluorsave mountant.

The percentage survival of neurons fixed in culture and stained using Hoechst 33342 (100ng/ml) was similar to counts of live neurons in culture hence the latter method was preferred over Live/Dead assay. It meant the same neurons could be followed for a longer period of time especially as some pathways take longer to react.

2.2.4 AraC Optimisation

48-72 hours after the plating of dissociated SCG neurons for trial cell cultures, it became difficult to count viable growing neurons **(Fig 2.3; Panel A+B)** due to the proliferation and attaching glia.

Over the last decade, researchers have used cytosine arabinoside (-b-D-arabino-furanosylcytosine, AraC) as a way of inhibiting glial proliferation and growth in neuronal cultures of young mice. It functions as a DNA-damaging agent that interrupts DNA synthesis and kills all proliferating cells. However, DNA damage can also kill post-mitotic cells, such as neurons. *In vitro*, AraC is toxic to post-mitotic sympathetic neurons (Wallace and Johnson 1989; Martin et al., 1990), through activation of two separate cell death pathways (Besirli et al., 2003; Putcha et al., 2003). One pathway requires phosphorylation of c-Jun (Besirli et al., 2003; Putcha et al., 2003) followed by Bcl-2 family member Bax, which mediates mitochondrial cytochrome c release and caspase activation (as seen during neurotrophic withdrawal in neonatal neurons) (Martin et al., 1990). The second pathway is Bax-independent and is significantly delayed, similar to the delayed death observed in camptothecin-treated embryonic cortical neurons in the presence of caspase inhibitors or after Bax deletion (Stefanis et al., 1999).

Importantly, (Besirli and Johnson 2003) discovered that DNA-damage induced c-Jun expression and phosphorylation, in P0-P1 rats, was not disturbed during JNK inhibition, thus confirming that AraC-induced cell death of neurons is JNK-independent. This supported a study indicating that apoptosis in sympathetic neurons is induced via a p53-dependent, JNK-independent, mechanism (Anderson and Tolkovsky 1999). However, these experimental culture studies were performed on developing neurons so, for the ensuing work, it was important to find the optimal concentration for use on ageing neurons without a detrimental effect to the survival and growth in culture.

To determine this, AraC was administered at different concentrations to NGF-treated cultured aged SCG neurons (**Figs 2.2**). In the past, 10μM AraC had been applied to younger neuron cultures without noticeable damage to neurons (Orike et al., 2001a), so that gave an indication into the concentrations that should be used in this experiment. The growth and survival of aged SCG neurons was monitored for three days. After 48 hours (**Fig 2.2),** the NGF-mediated neuronal response began to decline in the presence of 10μM AraC, although neuronal growth and survival became significantly reduced at a concentration of 30μM AraC. In the presence of 1μM and 3μM AraC, the survival and neurite outgrowth of aged SCG neurons, compared to control, was not significantly different.

The effect of AraC on the morphology of glia in culture was also observed (**Fig. 2.3**). In the absence of AraC, there was extensive glial proliferation and process outgrowth (**Fig 2.3; A+B**). However, the addition of AraC restricted this physiological response to NGF, and the strength of this restriction correlated with an increase in AraC concentration, with no glial proliferation and process outgrowth in the presence of 30μM AraC.

At a concentration of 3μM AraC, glial proliferation and process outgrowth was sufficiently restricted and the survival and growth of neurons was maintained. Therefore 3μM AraC provided an ideal concentration to limit the proliferation of glia without a detrimental effect to aged neurons, and without compromising the experiment. The same results were observed 72 hours after plating and there was no visible effect of AraC on glia after 24 hours in culture (results not shown).

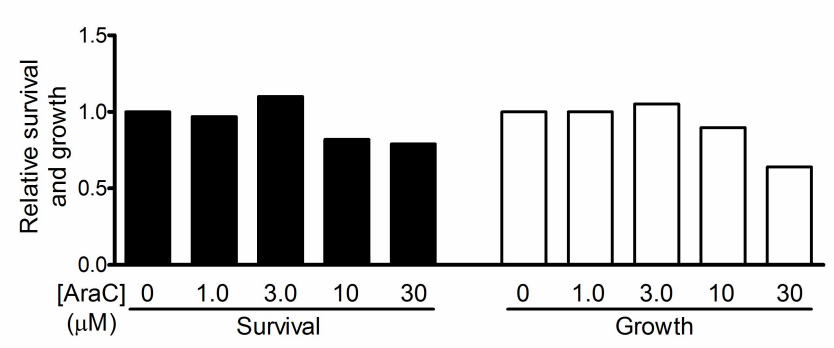


Figure 2.2: Concentration dependent effect of AraC on growth and survival of aged SCG neurons

After 48 hours in culture, the addition of 1μM, 3μM and 10μM AraC did not significantly affect the survival and neurite outgrowth of aged SCG, relative to control. However, there was a significant effect in the presence of 30μM AraC and growth and survival of aged SCG neurons is reduced ($p<0.0001$). This was representative of three experiments.

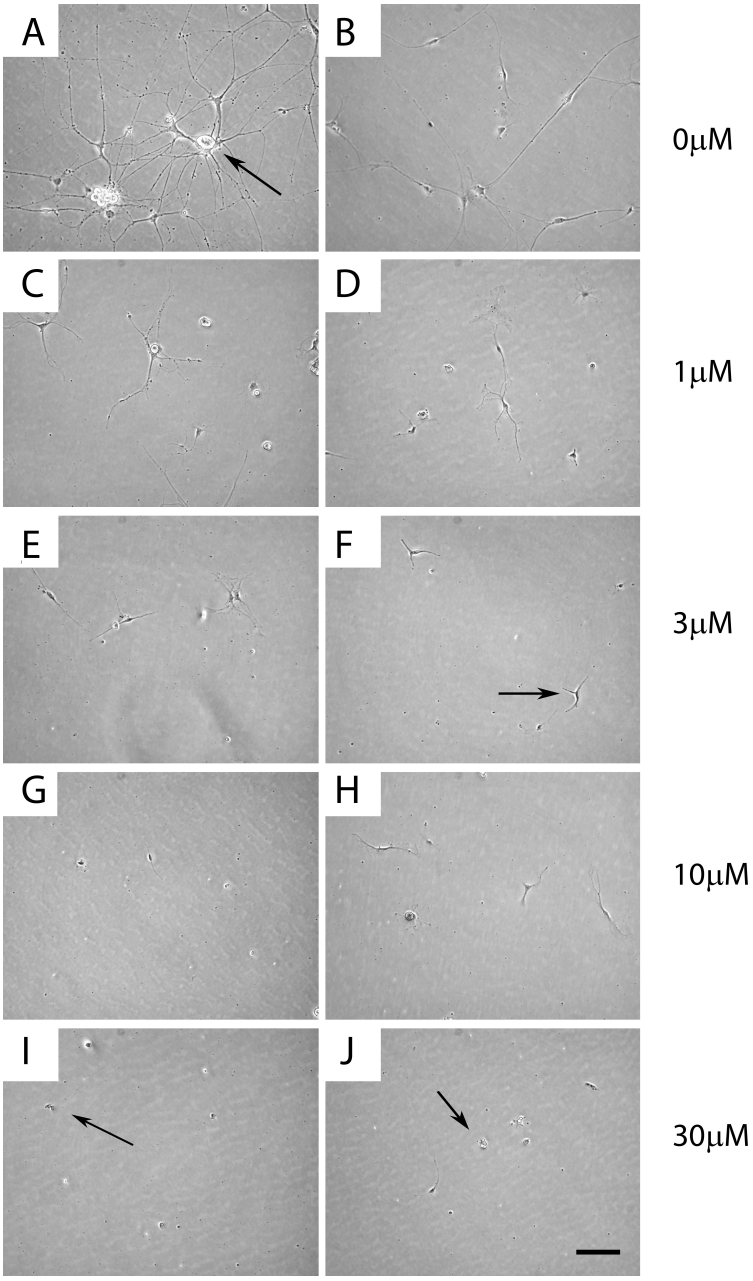


Figure 2.3: Representative phase contrast images showing the concentration dependent effect of AraC on the growth of glia in aged SCG cultures

(A+B) 0μM AraC, (C+D) 1μM AraC, (E + F) 3μM AraC, (G+H) 10μM, (I + J) 30μM. After 48 hours in culture, there was clearly strong outgrowth of glial processes in the absence of AraC, with many wrapping around neurons (arrow A). The addition of AraC restricts glial process outgrowth and proliferation and this became more pronounced with an increase in concentration (C-H). Complete restriction was observed at 30μM (I & J) (arrow J). Scale bar=100μm

Experimental studies were also conducted to assess whether AraC was having an indirect impact on cell survival or growth of aged sympathetic neurons through the inhibition of glial proliferation or process outgrowth in culture. In aged SCG neuron cultures containing NGF and JNK inhibitor e.g. **JNK inhibitor VIII (Fig 2.4)**, the survival and the proportion of growing neurons did not change significantly, following the addition of AraC, compared to control on Day 1. The equivalent was reported on Day 3. Therefore, AraC was included in all cultures containing JNK inhibitors.

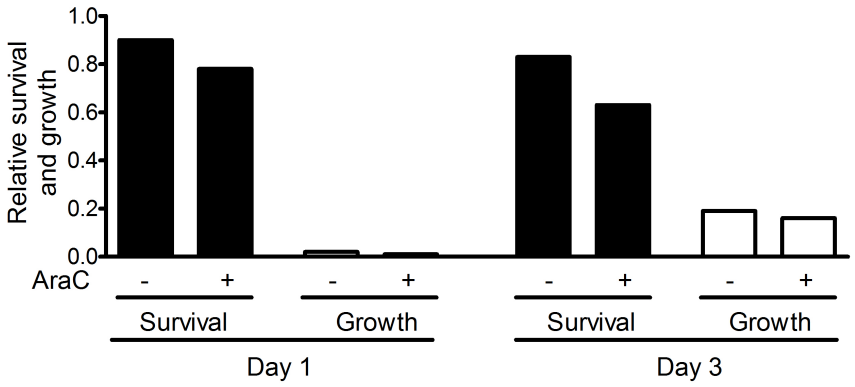


Figure 2.4: Effect of AraC on NGF and JNK Inhibitor VIII-treated aged SCG neurons

All SCG cultures derived from aged mice contained NGF and JNK inhibitor VIII (10μM) in the presence or absence of 3μM AraC. AraC did not affect the neurite outgrowth or survival of NGF and JNK Inhibitor VIII-treated aged SCG neurons on Day 1 or Day 3. Representative of three experiments (n=300).

2.3 Neuron and Glia Differentiation

Neurons and glia are clearly distinguishable in culture but for clarity, cell-type identity was confirmed by immunohistochemical analysis. Previously cultured glial cells from mouse SCG have been stained with glial fibrillary acidic protein (GFAP) to identify glia (de Almeida-Leite and Arantes 2010). Therefore, the same marker was used here, as well as Tuj-1, a neuron specific marker, in dissociated SCG cultures from aged mice **Fig 2.5**.

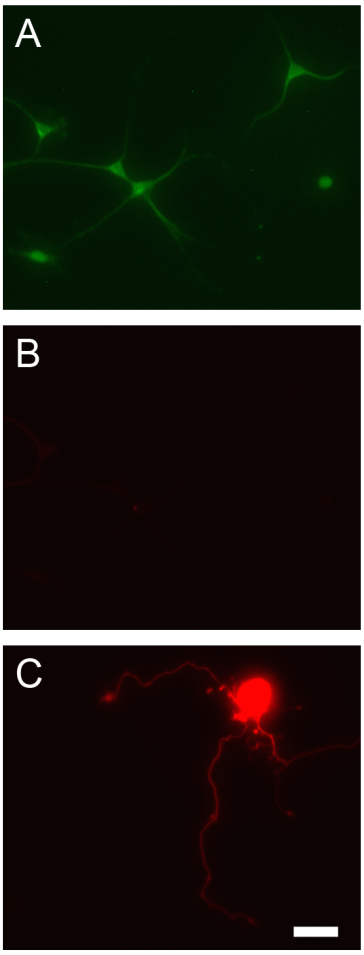


Figure 2.5: Immunohistochemical analysis to confirm neuron and glia identification

Dissociated SCG neurons cultured from aged mice stained with GFAP and Tuj-1. Glia were (A) GFAP positive and (B) Tuj-1 negative. (C) a growing neuron stained with Tuj-1. Scale bar = 50μm

2.4 Western blot Analysis

SCG tissues were dissected from F1 hybrid mice (n=3) and snap-frozen in liquid nitrogen. Samples were pooled (6 SCG=approx 3mg) then sonicated by six, one second bursts at an amplitude of 10 microns, on ice, and lysed in cold homogenisation buffer (HB) (200mM Tris pH7.4, 2mM ethylenediaminetetraacetic acid (EDTA) pH7.4, 2mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) pH 7.4, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, 1.09g sucrose, sodium vanadate), supplemented with protease inhibitor cocktail (1 tablet/ 10 mL, Roche Molecular Biochemicals, Mannheim, Germany). For pooled samples 60-70μL HB buffer was added. Brain samples were homogenised in cold HB buffer. Lysates were spun at 14600g at 4°C for 15 minutes. Western blot analysis was carried out using standard procedures, as previously described (Al-Shawi et al., 2008).

Briefly, 25μg of protein extract, in a sample buffer containing a final concentration of 4% β-mercaptoethanol, was loaded on a 1.5mm x 15 well 4-12% Bis-Tris polyacrylamide gel (Invitrogen) and was run using 4-Morpholineethanesulfonic acid (MES) SDS buffer (50mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA pH 7.3) at 95V for between 1 hour 45 minutes and 2 hours 20 minutes, on ice. Proteins were transferred to a nitrocellulose membrane (Invitrogen) using an immunoBlot (Invitrogen) at 100V for 7 minutes. Non-specific protein interaction was prevented by incubating the membrane with 5% dried skimmed milk powder (Marvel, Sainsbury's) dissolved in Tris-buffered saline (TBS) with 0.1% Tween-20 (Sigma) (TBS-T) for 1 hour at room temperature. After a series of washes in TBS, the membrane was incubated with primary antibody overnight at 4°C. Antibodies (**see 2.6**) were dissolved in 5% Marvel and TBS-T. The membrane was subsequently washed and incubated with goat anti-mouse or goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz, 1:5000) for 1 hour, at room temperature. Signal detection involved incubating the membranes with ECL-plus reagent (GE Healthcare) for 1 minute, then placing the membrane against a radiographic film before developing at different exposure times.

To control for variation in protein loading, Western blots were stripped prior to re-probing with anti-actin (clone 4 MAB1501, Millipore, UK) at a dilution of 1:2000. Secondary antibodies were administered at a dilution of 1:1000 (goat anti-rabbit Ig-HRP, Dako, UK) or 1:2000 (rabbit anti-mouse Ig-HRP, Dako, UK) for 1 hour RT.

2.5 Immunohistochemistry

For immunofluorescence analysis, cells in culture were washed three times in PBS, and fixed for 10 minutes with 4% paraformaldehyde. Coverslips were then dipped and rinsed three times in PBS again. 0.1% Triton X100 was applied to coverslips for 10 minutes at room temperature before washes were repeated. Cells were blocked for one hour, in PBS and 2% BSA, before removing and adding primary overnight at room temperature. Coverslips were washed 3 x 5 minutes in PBS. Detection was achieved using goat anti-rabbit or goat anti-mouse antibody (Alexa) at a dilution of 1:400; incubated for 1hr at room temperature. Three, 5 minute washes were followed by nuclear counterstaining with Hoechst 33342 100ng/ml. After two washes slides were mounted with CityFluor.

2.6 Antibodies

Table 2.3: Antibodies

Tuj-1 anti-neuron-specific Class III β-tubulin mouse monoclonal was kindly donated by Tim Cowen.

Antibody	Supplier	Concentration (μL)	Apparent size (kDa)	Expected size (kDa)	Method
mouse anti-hsp70	Enzo	1/1000 or 1/50 & 1/100	~62	70	Western/ Immuno
rabbit anti-TrkA	Chemicon International	1/1000	~98		Western
rabbit anti-sortilin	Abcam	1/2000	~90	95	Western
mouse anti-actin	Millipore, UK	1/2000	~40	38	Western
rabbit anti-NRAGE polyclonal	Upstate	1/500	~90	96	Western
anti-Tuj1		1/1000			Immuno
rabbit anti-GFAP polyclonal	Dako	1/1000			Immuno
rabbit anti-Neurofilament	Chemicon International	1/1000			Immuno

2.7 Live/Dead Glial Assay

Primary aged SCG neurons, using the cell culture method above **(2.2)** were plated at 300/well and cultured for 48 hrs. To determine the ratio of live/dead glial cells, a Molecular Probes Live/Dead Viability/Cytotoxicity assay kit (L-3224) was used. Solutions were made up immediately prior to use with 1ml solution added to each well required for counting. Cells were incubated for 15 minutes at 37°C.

The two-colour fluorescence assay confirms the viability of cells through the determination of intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of intracellular esterase activity, which is verified by the enzymatic conversion of cell-permeant polyanionic dye calcein AM into calcein to produce a uniform green fluorescence (ex/em ~495 nm/~515nm). In contrast, ethidium homodimer-1 (EthD-1) enters cells with damaged membranes, and undergoes 40-fold enhancement of fluorescence after binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495nm/~635 nm). This does not occur in live cells because EthD-1 is excluded by the intact plasma membrane.

2.8 NRAGE siRNA delivery

The NRAGE sequence consisted of a 22 nucleotide (22nt) duplex with disulphide overhang, as described and validated by (Matluk et al., 2010). The NRAGE siRNAs (Dharmacon, UK) were chemically synthesised and received in lyophilised, pre-annealed duplex form. siRNA pellets were resuspended in sterilised RNAse-free water then incubated for 1 hour at room temperature before linkage to the vector peptide Penetratin-1 (Pen-1) (Davidson et al., 2004) (MP Biomedicals, UK) in non-reducing conditions. The final concentration of the solution was 80μM and kept at 4°C until use.

Sense sequence
S-S.G.G.C.U.U.G.G.A.A.U.G.A.C.A.C.U.A.C.U.U.U
Antisense sequence
A.G.U.A.G.U.G.U.C.A.U.U.C.C.A.A.G.C.C.U.U

A control sequence was generously donated by Elena Ribe Garrido. For delivery of siRNA, cells were cultured as described earlier (**2.2**). siRNA was heated to 65^0C for 15 minutes then 1 hour at 37^oC, 5% CO_2. At the time of plating, NRAGE siRNA and control siRNA containing Pen-1 was administered at 80nM.

2.9 Statistical analysis

Statistical analysis was performed using the Chi squared χ^2 test using GraphPad Prism 5.0, which evaluates the statistical significance of differences in frequencies of classes (e.g. live and dead) between samples. All comparisons were analysed within one experiment, i.e. limited to treated and untreated control populations of cells derived from a single preparation of neurons. Experiments were repeated in triplicate, and differences were not deemed to be significant unless there was concordance between the different experiments.

Quantitative analysis of western blot data was performed by densitometry of entire bands, with subtraction of background immediately adjacent to the band. The ratios of background-subtracted desired protein and actin signals from samples analysed on the same blot were analysed by the Mann-Whitney test using GraphPad Prism 5.0 to determine any significant difference in the relevant protein, with the threshold value $p=0.05$.

Quantitative immunofluorescence was performed as previously described (Al-Shawi et al., 2008); statistical analysis was executed by the Mann-Whitney test using GraphPad Prism 5.0, with the threshold value $p=0.05$.

Chapter 3
- RESULTS -

3.1 The effect of JNK inhibition on the growth and survival of ageing peripheral neurons

3.1.1 Introduction

3.1.1.1 JNK activation and regulation

JNKs are required for both neuronal apoptosis and axon degeneration, in response to stress, but also have the ability to regulate neuronal growth and homeostasis (Chang et al., 2003; Bjorkblom et al., 2005). Therefore, signalling diversity is vital for the execution of a number of different functions (see **Introduction 1.3.3**).

While the focus of this study remains with neurotrophic-induced JNK signalling in adult and aged peripheral neurons, it is important to understand that activation of JNK can be prompted by a response to many stimuli. Also termed stress-activated protein kinases (SAPKs), JNKs respond to external stresses such as heat shock (Adler et al., 1995), UV irradiation (Pulverer et al., 1991; Derijard et al., 1994) and osmotic and redox stress (Bogoyevitch et al., 1995). JNKs can also be activated during physiological responses via activation of G-protein-coupled receptors (GPCRs) and the non-canonical Wnt pathway (Marinissen and Gutkind 2001; Pandur et al., 2002).

3.1.1.2 JNK-mediated cell death in developing peripheral neurons

In cultured neonatal sympathetic neurons, NGF withdrawal causes $p75^{NTR}$-mediated neuronal apoptosis via JNK-dependent c-Jun activation (Estus et al., 1994; Ham et al., 1995; Eilers et al., 1998; Bruckner et al., 2001). To elicit this signal, $p75^{NTR}$-induced activation of JNK requires receptor interacting proteins e.g. NRIF (Linggi et al., 2005), TRAF6 (Yeiser et al., 2004), Rac (Harrington et al., 2002), NRAGE (Salehi et al., 2002).

Once activated, JNK binds the NH_2- terminal activation domain of the c-Jun transcription factor (Adler et al., 1992; Hibi et al., 1993) and also binds activating transcription factor (ATF2). Activated c-Jun/ATF2 dimers subsequently regulate the *Jun* promoter, which increases c-Jun levels and mediates cell death (Herr et al., 1994; Ham et al., 1995; Yuan et al., 2009). This prompts the expression of pro-apoptotic genes *Dp5, Bim* and *Puma,* which are apoptotic facilitators of the Bcl-2 protein family (Harris and Johnson 2001; Whitfield et al., 2001; Putcha et al., 2003; Besirli et al., 2005; Jacobs et al., 2005; Wyttenbach and Tolkovsky 2006; Kristiansen et al., 2011). Induction of these BH3 domain-only proteins activate the intrinsic apoptotic cascade (Chen and Tan 2000; Tournier et al., 2000), and involves the release of several pro-

apoptotic factors, including cytochrome c and SMAC, through the permeabilisation of the outer mitochondrial membrane. These factors co-operate to activate initiator caspase-9, which mediates the cleavage of several cellular targets during apoptosis. In SCG neurons, trophic deprivation stress and activation of the $p75^{NTR}$ receptor leads to BAX-dependent release of cytochrome *c* and apoptotic death (Putcha et al., 2003). Although the death function of these proteins is executed in the cytoplasm, the initial trigger for the rapid induction of BH3-only proteins occurs in the nucleus and is JNK-dependent (Putcha et al., 2003). Importantly, the role of c-Jun in mediating JNK-dependent neuronal death has not been demonstrated across all models (Okuno et al., 2004; Bjorkblom et al., 2008; Ruff et al., 2012) suggesting JNK is not always involved in c-Jun-mediated death and conversely, that c-Jun is not always activated by JNK.

Additional gene targets for JNK have been discovered in developing sympathetic neurons deprived of NGF, which suggests a completely independent regulatory pathway to JNK-c-Jun in the process of apoptosis. These include the ER stress pathway genes *Trib3* (tribbles homologue 3), *Ddit3* (DNA damage-inducible transcript 3) and *Txnip* (thioredoxin interacting protein), among others (Kristiansen et al., 2011). In cultured developing sympathetic neurons, the Rac GTPase target POSH has also been associated with JNK-induced neuronal death through NGF deprivation (Tapon et al., 1998; Xu et al., 2003).

3.1.1.3 JNK-mediated growth

Experiments concerning JNK actions on neurite outgrowth have largely been performed in PC12 cells (Heasley et al., 1996; Eilers et al., 1998; Waetzig and Herdegen 2003a; Eom et al., 2005), which are ''neuronally'' differentiated by NGF and compounds or constructs that simulate NGF treatment (Yao et al., 1997; Goodman et al., 1998).

Through the use of pan-JNK inhibitors (SP600125) and transfection with dominant negative JNK mutants, which substantially decrease sprouting (Kita et al., 1998; Giasson et al., 1999; Waetzig and Herdegen 2003a), NGF-induced neuronal growth in PC12 cells was shown to display JNK dependency (Kobayashi et al., 1997; Goodman et al., 1998). Further studies confirmed that all JNK isoforms are involved in neurite outgrowth (Yang et al., 1998; Waetzig and Herdegen 2003a; Waetzig and Herdegen 2003b; Waetzig and Herdegen 2005), although JNK3-dependent NGF-induced

sprouting in PC12 cells requires transfection of exogenous JNK3 (Waetzig and Herdegen 2003b). The idea that neuritogenesis is supported by all JNK isoforms is reinforced by studies in primary hippocampal and cortical neuron cultures (Eminel et al., 2008), however these experiments were not performed in the presence of NGF.

Cell culture studies have further reported a high enrichment of activated JNK in axons; signifying a role for JNK in axonogenesis. Additional studies indicate JNK dependency in neural migration, polarity, and axon guidance, although once again, experiments have predominantly focused on central neurons during early development, and it is not known whether this is NGF-induced (Coffey et al., 2000; Oliva et al., 2006; Tararuk et al., 2006; Qu et al., 2013). Recently, Qu et al., (2013) demonstrated that JNK signalling is required for Netrin signalling (axon guidance) in the developing nervous system. Netrin-1 increased JNK1, not JNK2 or JNK3, activity in the presence of Netrin receptors.

Cytoplasmic JNK substrates like doublecortin (DCX), microtubule assembly-promoting proteins (MAP1B, MAP2) and the heavy unit of the neurofilament protein (NFH) are also important for mediating neurite elongation, as observed in cell culture studies of the developing brain (Kawauchi et al., 2003; Waetzig and Herdegen 2003a; Gdalyahu et al., 2004). Again, JNK1 has been singled out to have a more distinguished role in these physiological processes (Chang et al., 2003; Kawauchi et al., 2003; Bjorkblom et al., 2005). In neural migration, Westerlund et al., (2011) discovered that JNK phosphorylation of SCG10, a stathmin brain-derived JIP, is a key mechanism that controls the rate of neuronal cell movement in corticogenesis. This process is also facilitated by JNK1, unlike after NGF deprivation in developing sympathetic neurons, where phosphorylation of SCG10 is controlled by JNK3 (Neidhart et al., 2001).

Further downstream, while c-Jun is involved in JNK-dependent cell death, it is also necessary for the formation of neurites (Leppa et al., 1998; Dragunow et al., 2000; Levkovitz and Baraban 2002). Overexpression of c-Jun mimics some effects of NGF and leads to neurite outgrowth (Leppa et al., 1998; Dragunow et al., 2000). However, c-Jun overexpression-mediated differentiation does not result in the entire range of differentiation characteristics displayed in NGF-treated PC12 cells (Dragunow et al., 2000); suggesting that c-Jun-triggered gene expression is sufficient for sprouting, but not for a complete differentiation process.

It is important to mention that the nuclear activity of JNKs is different in neuronal cell lines and primary cells: in PC12 cells, NGF-induced neuritogenesis is substantially mediated by phospho c-Jun (Leppa et al., 1998; Dragunow et al., 2000); in primary central neurons, phosphorylation of c-Jun is down-regulated despite an increasing activity of JNK (Coffey et al., 2000; Eminel et al., 2008).

Overall, NGF promotes a multitude of actions on peripheral axons *in vitro* including elongation, branching, growth cone turning responses, and changes in growth cone morphology (Gallo and Letourneau 1998; Markus et al., 2002), all of which may be required for NGF-mediated innervation of final target tissues *in vivo.* To facilitate these processes, several studies in PC12 cells propose that JNK works in tandem with kinases, PI3K and ERK1/2, in the complete response to NGF in neuritogenesis (Kobayashi et al., 1997; Kita et al., 1998; Zentrich et al., 2002; Waetzig and Herdegen 2003a; Xiao and Liu 2003). Research indicates that PI3K induces sprouting through the activation of JNKs, whereas for neurite outgrowth, ERK1/2 and the JNKs signal in concert (Kobayashi et al., 1997; Kita et al., 1998; Zentrich et al., 2002; Waetzig and Herdegen 2003a; Xiao and Liu 2003). It has been further suggested that ERK1/2 trigger the early stages of sprouting, whereas JNKs are responsible for later phases of neurite formation and stabilisation (Xiao and Liu 2003).

3.1.1.4 JNK signalling in ageing and after injury

As previously discussed (**Introduction 1.4**), nerve injury triggers a number of changes within the affected neurons and surrounding non-neuronal cells that ultimately result in successful target re-innervation or cell death, though this is delayed in ageing. The morphology of sympathetic neurons, axons and dendrites, their targets undergoing atrophy in ageing (Gavazzi et al., 1992; Gavazzi and Cowen 1993a), and the benefits of NGF in these areas, have been significantly researched (Andrews and Cowen 1994b; Thrasivoulou and Cowen 1995; Cowen et al., 1996; Cowen et al., 1997). Although regeneration of aged sympathetic neurons can occur in the absence of NGF (Gloster and Diamond 1992), collateral sprouting is NGF-dependent and can be affected in ageing due to an impaired responsiveness to NGF (Kuchel 1993; Gavazzi 1995). The precursor to NGF, proNGF, has also displayed neurotrophic properties in aged sympathetic cultures (Fahnestock et al., 2004; Al-Shawi et al., 2008), however further studies are required to determine whether this is JNK-dependent **(Results 3.3**).

While further studies are required in sympathetic neurons, JNK actions have already been identified in the process of regeneration through *in vivo* studies on sensory neurons, derived from adult rodents (Kenney and Kocsis 1998; Barnat et al., 2010; Ruff et al., 2012). In the study undertaken by Ruff et al., (2012), c-Jun is shown to promote peripheral nerve regeneration after facial nerve cut in 8-10 week old mice, although it is unknown whether this is NGF-dependent. *In vivo* and *in vitro* studies by Lindwall et al., (2004), also on rat sensory neurons, reported that JNK inhibition substantially reduces axonal outgrowth, c-Jun activation and ATF-2 induction, without affecting survival.

As mentioned, neuritogenesis is reliant on JNK2 and JNK3, but not JNK1 during the axonal regeneration of adult mouse DRG. However, sustained neurite elongation requires JNK1 and JNK2. JIP1 is also involved in the initiation and extension stages; Barnat et al., (2010) discovered that JIP1, as well as activated JNKs, were present in the cytoplasm of sprouting or regenerating axons, suggesting a local action on cytoskeleton proteins. In the same study, JNK inhibition-induced neurite retraction was also shown to involve MAP1B, an important microtubule regulator involved in the elaboration and maintenance of axonal and dendritic processes**.** Further reports revealed that JNK1 and JNK2 control the phosphorylation state of MAP1B. Therefore, signalling by individual JNKs is differentially implicated in the reorganisation of the cytoskeleton, and neurite regeneration.

3.1.1.5 Cytoskeletal functions

It has become clear that the role of JNKs in neurite elongation and nerve fibre regeneration is dependent on the stability and reactive flexibility of the cytoskeleton. A complex network of interlinking tubules and filaments, the cytoskeleton has a heavy influence on the intracellular transport of vesicles by molecular motor proteins such as kinesins, dyneins, and myosins. Studies by Middlemas et al., (2003) in peripheral neurons show that not only is JNK localised to nerve axons, but it is transported both anterogradely and retrogradely. Further investigation by Cavalli et al. (2005), in the sensory neurons of adult rodents, demonstrated that after nerve injury, JNK is activated locally as part of a retrograde axonal damage signal and that Sunday Driver (syd), a receptor protein homologous to JIP3 (Bowman et al., 2000), interacts with both JNK3 and kinesin-I as a scaffolding protein. In turn, this may mediate both the anterograde and retrograde transport of JNK via interaction with

kinesin-I and dynactin/dynein, respectively. As anterograde transport is disrupted and retrograde transport increases following axotomy-induced JNK3 activation, it is believed that JNK3 acts as a surveillance sensor that responds to stress by altering motor transport.

It has been suggested that distinct outcomes of positive and negative influences on neuronal migration may be attributed to opposing functions of nuclear and cytosolic JNK (Bjorkblom et al., 2008). The same group further propose that simultaneous knockdown of all three JNK isoforms is not enough to provide neuroprotection. Bjorkblom et al. (2008) argue that JNK-dependent death responses are location specific rather than isoform specific, and that JNK inhibition in the cytosol does not protect from trophic withdrawal-induced death in early development, but it does in the nucleus.

3.1.1.6 Drug Therapy and Experimentation

In order to understand the functional importance of JNK, researchers have benefited from molecular cloning, and experiments to decipher the structural properties of the different JNK proteins (Xie et al., 1998; Heo et al., 2004; Shaw et al., 2008). These, combined with several *in vivo* genetic disruption methods and screenings to identify small molecule ATP-competitive inhibitors such as SP600125 (anthrax(1,9-cd) pyrazole (2H)-0ne) (Bennett et al., 2001) have improved our understanding into the role of JNKs. Pan-JNK inhibitors, such as cell permeable peptides D-JNK-1 (XG-102) (Bonny et al., 2001), a retroinverso peptide derived from the inhibitory region of JIP-1, and SP600125 (Bennett et al., 2001) (JNK1,2 and 3; IC50 0.11, 0.11, 0.15μM respectively) have been used extensively in research for over a decade.

D-JNKI-1 was found to be neuroprotective during ischemia (stroke model) (Vaslin et al., 2011) and the kainic acid-induced seizure model in rat (Spigolon et al., 2010). The JNK3 pool in mitochondria was suggested as the D-JNKI-1 target responsible for neurodegeneration in the seizure-induced model in the hippocampus (Zhao et al., 2012). D-JNK1-1 has also been shown to have protective effects in animal models of AD (Ballatore et al., 2007; Tran et al., 2012).

More recently, it was demonstrated that p38α, and not JNK, was a potential target of D-JNKI-1 *in vivo* (Kaoud et al., 2011). Part of the same MAPK family as JNK, p38 is involved in maintaining synaptic plasticity in the CNS, and so selectivity against this

kinase could be misleading. Pan-JNK inhibitor, SP600125, is selective against p38b (Bennett et al., 2001) and is neuroprotective in transient brain ischemia/reperfusion-induced neuronal cell death in rat hippocampus (Guan et al., 2005).

SP600125 has also been found to provide neuroprotection in neurodegenerative diseases (0.3μM), such as HD (Reinhart et al., 2011) and a brain slice-based model of AD (Braithwaite et al., 2010). In adult rat, 10μM SP600125 strongly reduces JNK-dependent outgrowth of sensory nodose ganglion and DRG neurons while at 200μM, neurite outgrowth is completely blocked (Lindwall et al., 2004; Barnat et al., 2010). This can also be observed in sympathetic neurons (Lindwall and Kanje 2005). SP600125 inhibition or transfection with dominant negative JNK mutants also influences a reduction in NGF-induced sprouting in PC12 cells (Kita et al., 1998; Giasson et al., 1999; Waetzig and Herdegen 2003a). Indirect JNK inhibition through CEP-1347, a selective mixed lineage kinase (MLK) inhibitor (Xu et al., 2001), in cultured mouse sympathetic neurons, diminished phosphorylation of c-Jun and apoptotic death in response to NGF deprivation (Bruckner et al., 2001).

The notion that improved neuroprotection could be achieved through selective inhibition of JNK isoforms has further driven the search for isoform-specific inhibitors in the brain (Coffey et al., 2002; Brecht et al., 2005). In ageing, there is a lot of interest surrounding the discoveries of neuroprotection in JNK3 KO animals. In excitotoxic and ischemic models in the brain, JNK3 has been found to play a major role (Brecht et al., 2005). In 2003, in the attempt to find efficient and innocuous compounds to target JNK3, four crystal structures of JNK3 in complex with 2 different classes of inhibitors were reported. This formed a platform upon which modifications could be made in order to tackle issues such as potency and selectivity of inhibitors used. Early drug discovery has been reviewed by Siddiqui and Reddy (2010), and any inhibitors identified prior to 2010 have been reviewed by Bogoyevitch and Arthur (2008); Bogoyevitch et al., (2010). Each review promotes the idea that isoform-specific inhibition of JNK and, more specifically, the development of a selective JNK3 inhibitor is of paramount importance for the treatment of neurodegenerative diseases (Resnick and Fennell 2004).

3.1.2 Aims

While the effect of JNK inhibition on survival and growth has been monitored in adult sensory neurons following injury, little is known about the effect in adult sympathetic neurons, let alone aged sympathetic neurons.

The hypothesis that will be addressed in this section is that JNK is important for the survival and growth of explanted SCG neurons from young adult and aged mice.

The effect of JNK inhibition on SCG neurons will be monitored in culture, but it will be important to observe whether there are differences between young adult and ageing neurons. The addition of isoform-specific inhibitors will also seek to establish whether specific JNK isoforms are important for different neuron survival and growth responses. For inhibitors used, please see **Table 2.2 p42**.

3.1.3 Results

3.1.3.1 JNK inhibition in young adult and aged SCG neurons

To study the effect of JNK inhibition on peripheral neurons of young adult and aged mice, pan-JNK inhibitors were applied to dissociated SCG sympathetic neurons in culture. Two inhibitors with different modes of delivery were administered: **JNK inhibitor II**, an ATP competitive inhibitor of JNK and **JNK inhibitor III**, a cell permeable peptide, which disrupts the c-Jun/JNK interaction.

Initially, pan-JNK inhibitor, **JNK Inhibitor II (SP600125** as it is more commonly known), was administered at 10μM to NGF (0.4nM)-treated and untreated control SCG neuron cultures derived from young adult and aged mice.

JNK Inhibitor II did not significantly affect survival, either in the presence or absence of NGF (**Fig 3.1**). However, neurite outgrowth of SCG neurons isolated from both young adult and aged mice was significantly reduced. The proportion of growing neurons compared to control cultures containing **NGF and JNK inhibitor II** decreased by 90%.

As the effects of JNK inhibition on survival were similar with the addition of NGF to culture **(Fig 3.1)**, any further experiments shown are confined to neurons cultured in the presence of NGF.

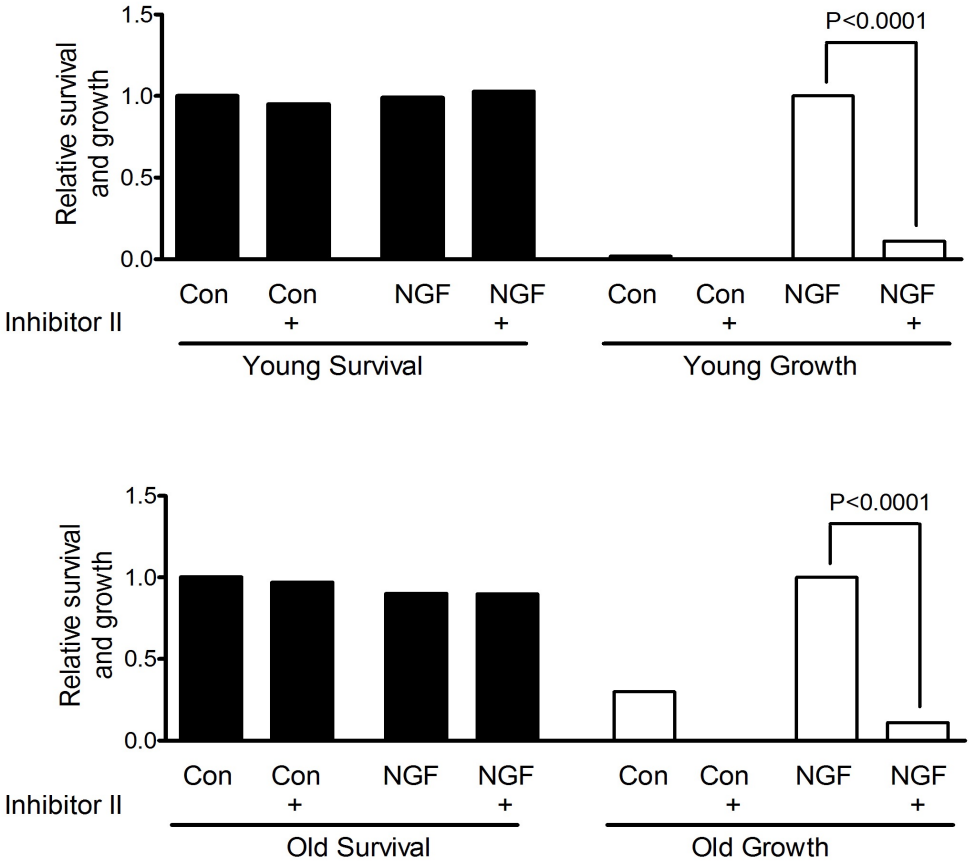


Figure 3.1: Effect of JNK Inhibitor II on survival and neurite outgrowth of cultured young adult and aged SCG neurons

Relative survival (filled bars) and growth (open bars) of neurons from young adult and aged mice is shown after culture under the conditions indicated. For clarity, survival was normalised to the control cultures; growth was normalised to NGF (0.4nM)-containing control cultures. JNK inhibitor II (10μM) did not significantly affect the survival, but did reduce the proportion of growing neurons ($p<0.0001$; $n=300$) in untreated and NGF-treated young adult and aged SCG neuron cultures.

To observe whether different mechanisms of action affect the outcome of JNK inhibition in aged mice, **JNK inhibitor III** was administered in a separate experiment.

Following the administration of **JNK inhibitor III** in cell culture**,** similar results were observed to previous experimental cultures containing **JNK Inhibitor II,** whereby **JNK inhibitor III** did not affect the survival of young adult and aged SCG neurons but it did restrict neuritogenesis **(Fig 3.2)**.

In **Fig 3.2**, pan-**JNK inhibitor III** reduced the proportion of growing NGF-treated aged SCG neurons by approximately 70% relative to NGF control. The proportion of growing NGF-treated SCG neurons was reduced by 40%, relative to control.

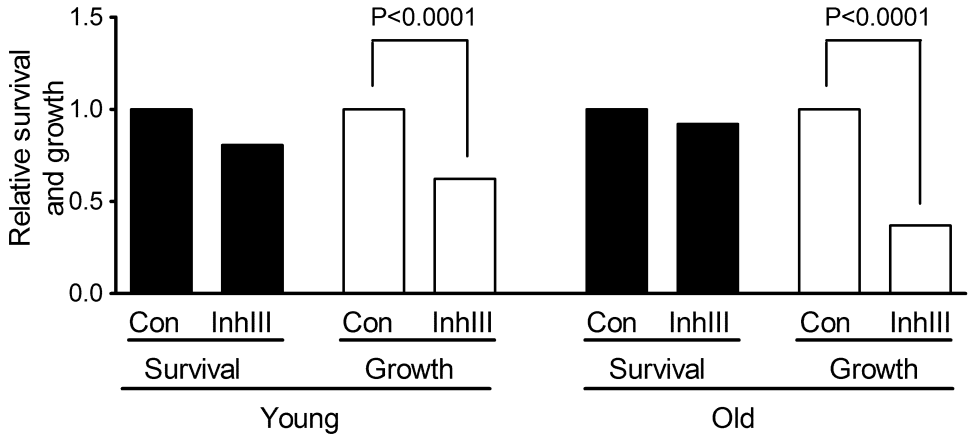


Figure 3.2: Effect of JNK Inhibitor III on the survival and neurite outgrowth of cultured young adult and aged SCG neurons

JNK inhibitor III (10μM) did not significantly affect the survival of NGF-treated young adult and aged SCG neurons, but significantly reduced the proportion of growing NGF-treated neurons in culture ($p<0.0001$; $n=300$).

To delve deeper into JNK dependency, specific inhibitors were administered to determine whether any specific JNK isoform has a greater impact on survival and neuritogenesis of SCG neurons derived from young adult and aged mice. **JNK inhibitor VIII** (inhibits JNK 1+2 more effectively that JNK 3) and **JNK inhibitor IX** (inhibitor of JNK 2+3) were administered at 10μM to NGF-treated SCG neurons from young adult and aged mice.

As expected, neuronal survival was not significantly affected by the addition of **JNK inhibitor VIII and IX** to NGF-treated cultures of young adult and aged SCG neurons **(Fig 3.3),** and a similar level of survival was observed compared with control-untreated cultures.

However, consistent with other JNK inhibitors used in this study, **JNK inhibitor VIII** and **JNK inhibitor IX** significantly reduced NGF-induced neurite outgrowth in young adult and aged cultured SCG neurons, although **JNK inhibitor IX** was much more effective.

JNK Inhibitor VIII reduced the proportion of growing NGF-treated young and aged SCG neurons by approximately 50% relative to NGF control. Whereas neurite outgrowth of SCG neurons is completely abolished in the presence of **JNK inhibitor IX** in both young adult and aged neurons, with double the reduction induced by **JNK inhibitor VIII**.

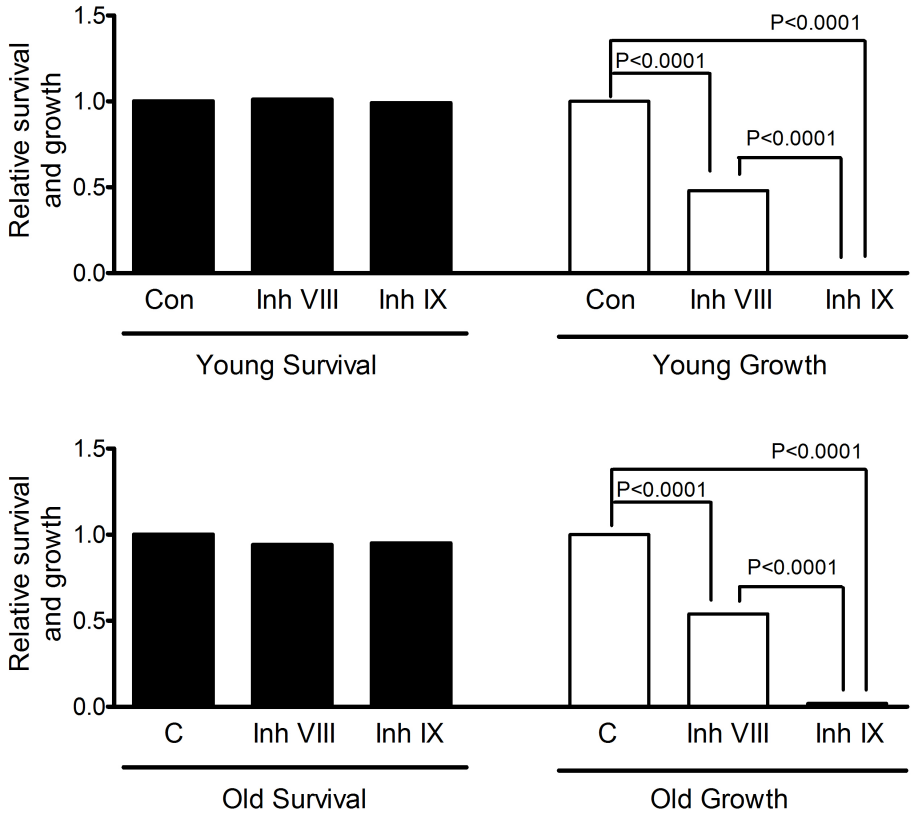


Figure 3.3: Effect of JNK inhibitor VIII and IX on the survival and neurite outgrowth of cultured young adult and aged SCG neurons

JNK inhibitor VIII and IX had no significant effect on the survival of NGF-treated SCG neurons of young adult and aged mice but significantly reduced the proportion of growing NGF-treated (0.4nM) SCG neurons derived from young adult mice, ($p<0.0001$; $n=300$) and aged mice ($p<0.0001$; $n=300$). JNK Inhibitor IX was more effective than JNK Inhibitor VIII and completely halted any neurite outgrowth ($p<0.0001$; $n=300$).

AEG3482, a cell permeable imidathiadiazole, was discovered, out of 17,000 natural, synthetic and semi-synthetic compounds, to reduce $p75^{NTR}$/NRAGE/JNK-mediated cell death of PC12 cells, following NGF withdrawal (Salehi et al., 2006). As an indirect JNK inhibitor, **AEG3482** works by activating HSP90, which subsequently induces HSF-1 dependent expression of HSP70 mRNA, an anti-apoptotic protein that binds

and deactivates JNK. Therefore, it was relevant to explore the effect of this compound on survival and neurite outgrowth of young adult and aged dissociated SCG neurons.

Fig. 3.4, indicates that **AEG3482** did not have an impact on neuronal survival in both young adult and aged NGF-treated SCG neurons in culture. However, **AEG3482** had a restrictive effect on NGF-induced neurite outgrowth in young adult and aged cultured SCG neurons. In **Fig 3.4**, **AEG3482** reduced the proportion of growing NGF-treated neurons by 80% in young adult SCG cultures and 70% in aged SCG cultures.

It is not possible to compare the two experiments because each culture requires different preparation so there may be variability in the culturing of neurons from mice of different ages, however, the key point is that the same result is observed in young adult and aged cultures.

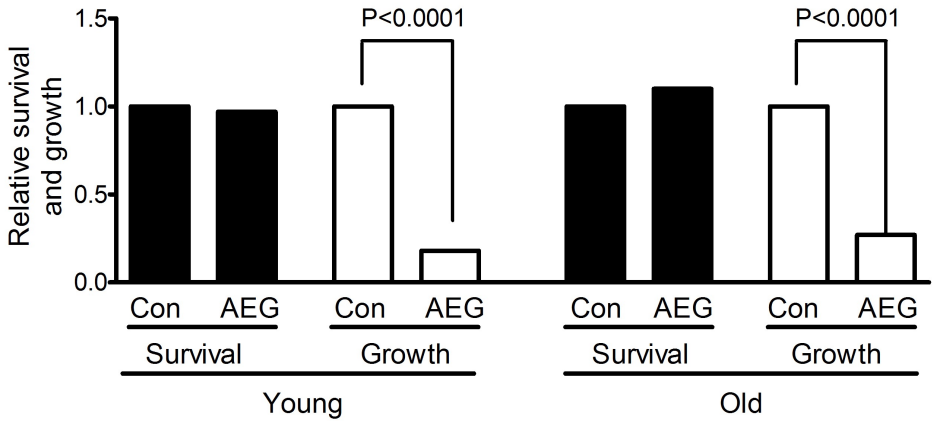


Figure 3.4: Survival and neurite outgrowth of AEG3482-treated cultured young adult and aged SCG neurons

AEG3482 (40μM) had no significant effect on the survival of NGF-treated SCG neurons of young adult and aged mice but significantly reduced the proportion of growing NGF-treated SCG neurons derived young adult mice ($p<0.0001$; $n=900$) and aged mice ($p<0.0001$; $n=900$).

Figure 3.5 gives a representative picture of how the neurite outgrowth of individual neurons was affected following the administration of different JNK inhibitors. NGF clearly had a positive effect on neurite outgrowth of aged neurons in culture (A). **JNK inhibitor II, III, VIII and AEG3482** (B-E) all restrict the proportion of growing cultured aged neurons, while **JNK inhibitor IX** (F) completely eliminates any NGF-induced neurite formation. Similar results were observed in young adult cultures (results not shown).

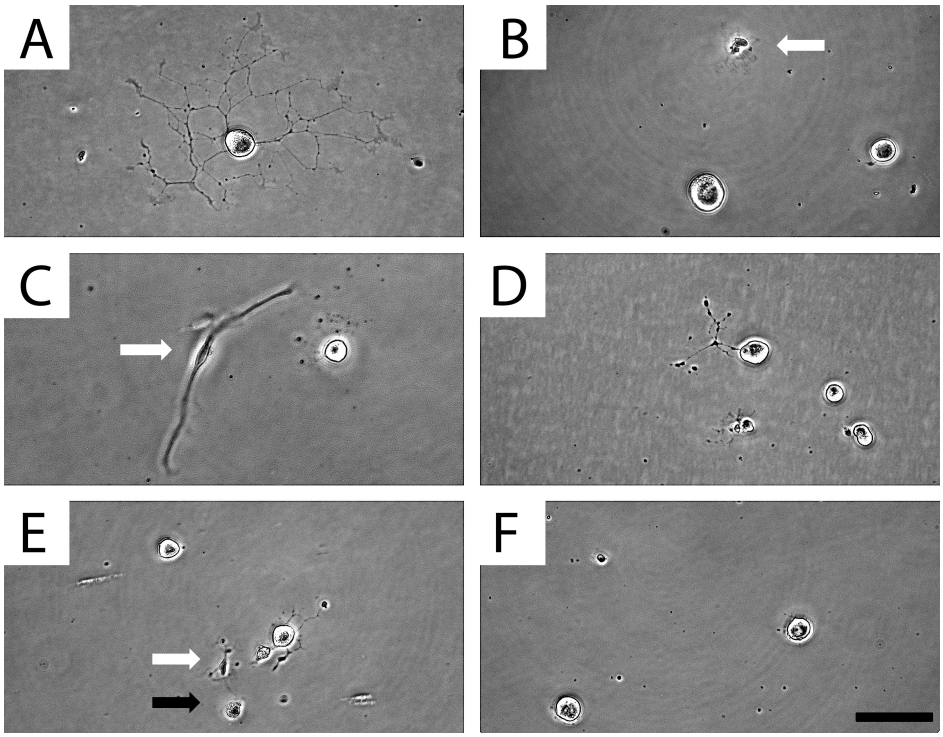


Figure 3.5: Representative photomicrographs of NGF-treated neurons in the presence of various JNK inhibitors in SCG cultures derived from aged mice.

A characteristic growing neuron cultured under NGF-treated control conditions. (B) NGF and JNK inhibitor II – treated culture, showing two living neurons with no neurite outgrowth and a glial cell (white arrow). (C) A neuron growing in the presence of NGF and JNK inhibitor III; the white arrow indicates a glial cell with extensions. (D) Neurons cultured with NGF and AEG3248, two of which show no growth. (E) Neurons cultured with NGF and JNK inhibitor VIII, one of which is dead (black arrow); nearby glial cell (white arrow). (F) Neurons cultured with NGF and JNK inhibitor IX, showing the typical absence of any significant growth. Scale bar = 100μm

3.1.3.2 HSP70 Immunohistochemistry and Western Analysis

In PC12 cells, **AEG3482** indirectly up-regulates the expression of anti-apoptotic protein HSP70, which peaks between 16-30 hours after **AEG3482** administration (Salehi et al., 2006). HSP70 also has the ability to restrict neuritogenesis; the use of HSP70 inhibitor, KNK437, in PC12 cells induced neurite outgrowth in the absence of stress stimulation (Koike et al., 2006).

As there was no difference in the survival and growth of neurons across ages in **AEG3482**-treated SCG cultures, young adult neurons were used to visualise HSP70 levels. Immunohistochemistry was carried out on fixed cultures, 21 hours after plating (**Fig 3.6)**. Plating density was approximately 600 cells/well and 20–30 pictures were taken of each condition. Rabbit neurofilament antibody (**Fig 3.6 a+d)** was applied to distinguish neurons from glial cells. The images are representative of each condition.

At a dilution of 1:50, neurons were clearly and intensely stained with HSP70 antibody. The data retrieved in control neurons (**Fig 3.6; A-C)** was fairly consistent unlike data from **AEG3482**-treated neurons (**Fig 3.6; D-F)**, which were slightly more variable. The neuron in **Fig 3.6; D** was much more intensely stained than neurons in **Fig 3.6; E + F**, and this variability was observed in a number of neurons within the population plated, under conditions where **AEG3482** was included in the medium. This was in contrast to the population of NGF-control treated SCG neurons, as represented in **Fig 3.6; A-C.**

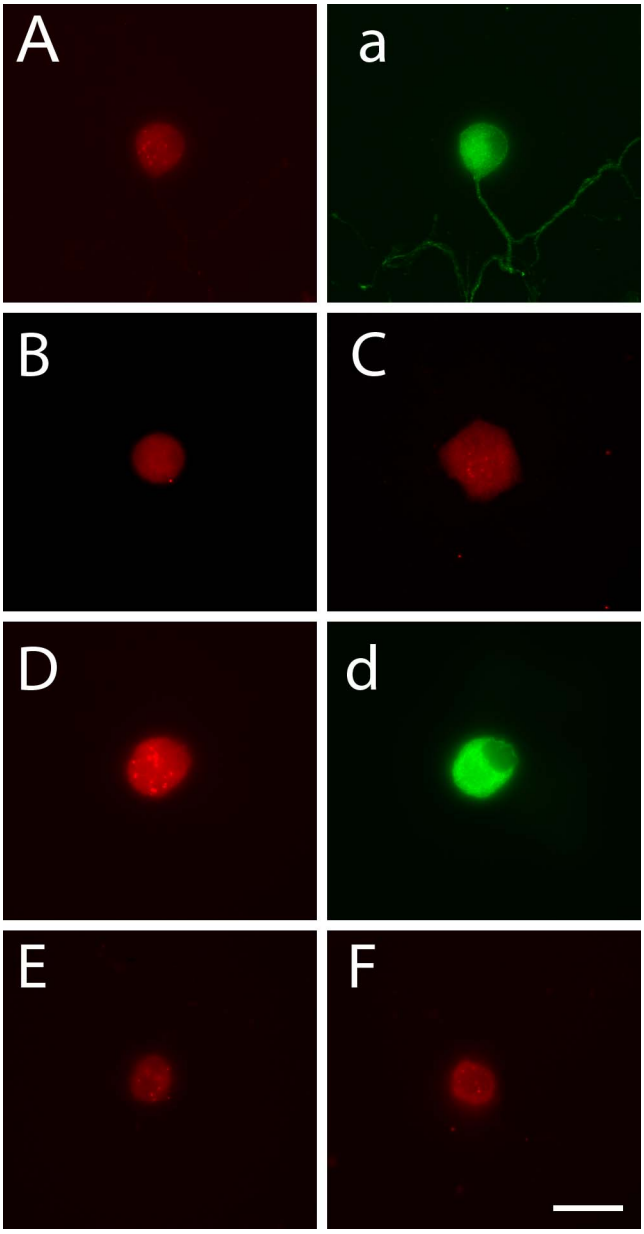


Figure 3.6: Representative photographs of HSP70 stained control-treated and AEG3482-treated young adult SCG neurons

(A, B and C) mouse HSP70 (mHSP70) stained SCG neurons (4 month) in NGF-treated control conditions. **(D, E and F)** mHSP70 stained cultured young adult neurons in NGF (0.4nM) and AEG3482 (40μM) treated conditions (1:50). **(a/d)** rabNeurofilament stained neurons (1:1000) corresponding to **A/D** panels. Neurons were fixed 21 hours after plating. Plating density: 600 cells per well. Scale bar = 25μm.

Although the analysis of the HSP70 staining intensities displayed no significant difference between control-treated young adult SCG neurons and neurons cultured in **AEG3482**, relatively large error bars in **(Fig 3.7)** confirm that the variability was high between individual neurons. The standard error for the control was 9.44 x 10^4. This was compared to 3.2 x 10^5 in **AEG3482**-treated SCG cultures.

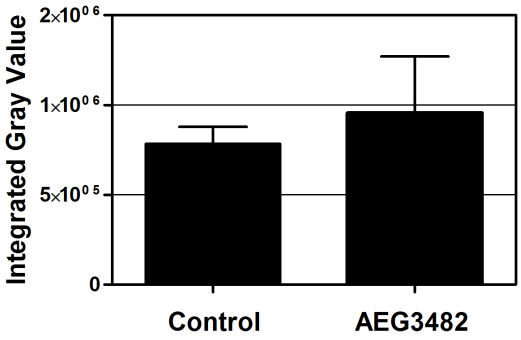


Figure 3.7: Effect of AEG3482 on HSP70 levels in young adult NGF-treated SCG neurons

Cultured 5-month old NGF-treated SCG neurons were fixed at 21 hours after plating. Neurons were stained with mHSP70 1:50. 20-30 pictures were taken of each condition then quantified using gray scale, as described in **Materials & Methods**. There was no significant difference between mHSP70 intensities of NGF-control treated neurons and neurons treated with NGF + AEG3482 (p=0.06). Standard error of Control: 9.44 x 10^4, AEG3482: 3.2 x 10^5.

To observe if there was a difference in HSP70 levels in sympathetic ganglia of young adult and aged mice, western analysis **(Fig 3.8)** was conducted on pooled SCG samples. Each lane represents a pool of six SCGs from three mice. Under conditions where noxious stimuli are absent, results indicated that there was no significant difference in HSP70 levels between age-groups.

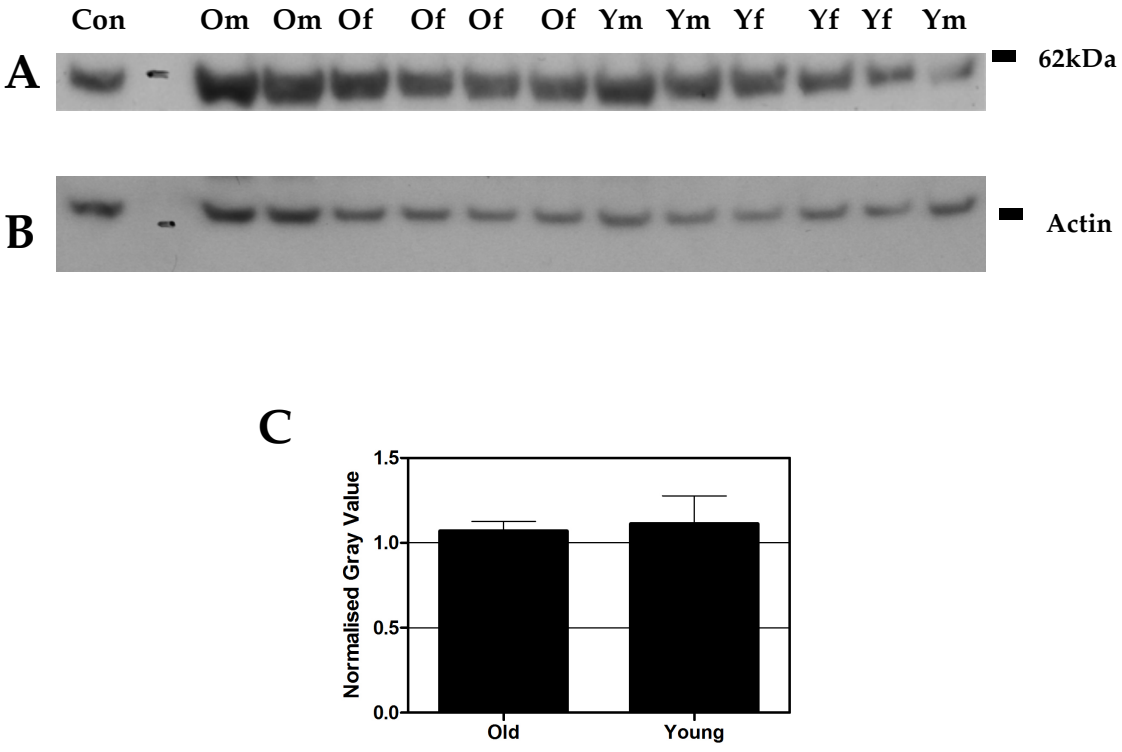


Figure 3.8: Western blot analysis of SCG lysates of aged and young adult mice

(A) HSP70, 62kDa **(B)** Actin, (**C)** There was no significant difference in HSP70 levels, normalised to actin, of SCG cells from old (24-36 months) and young adult (3-6 months) mice p=0.70. For each age-group, n=6, non-parametric t-test (Mann-Whitney). SCG lysates are represented by Om – old males, Of – old females, Ym – Young males, Yf - Young females. Con – brain tissue.

3.1.4 Discussion

The results from this chapter demonstrated that, while the survival of cultured young adult sympathetic neurons was unaffected by JNK inhibition, neurite outgrowth was reduced. The same pattern was also observed in aged SCG neurons suggesting that sympathetic responsiveness to JNK signalling is not impaired in ageing and correlates with previous studies that ageing neurons are not dependent on NGF for survival, but are for growth.

The application of pan-JNK inhibitors, **JNK inhibitor II and III**, which work through different modes of inhibition, ATP-competitive inhibition of JNK or disruption of the c-Jun/JNK interaction respectively, were able to produce similar results in short-term culture. Another compound used, which inhibits JNK indirectly, and was found to have an impact on neonatal neuron survival, is **AEG3482**. At 40μM, **AEG3482** reduced $p75^{NTR}$/NRAGE/JNK-mediated apoptosis of neonatal sympathetic neurons following NGF withdrawal (Salehi et al., 2006). When the same concentration was administered to NGF-treated young adult and aged cultured SCG neurons, in this study, **AEG3482** did not affect survival. Furthermore, results displayed a similar effect on neurite outgrowth as was observed following the addition of **JNK inhibitor II and III;** while there was no effect on survival, it was clearly evident that the **AEG3482** strongly inhibits NGF-induced neurite outgrowth of sympathetic neurons from young adult and aged mice.

In previous studies, **AEG3482** indirectly up-regulates the expression of anti-apoptotic protein HSP70, which peaks between 16-30 hours after **AEG3482** administration (Salehi et al., 2006) and rescues cells from $p75^{NTR}$-induced death. As cultured young adult and neurons are not dependent on NGF for survival, and as **AEG3482** had no affect on the survival of these NGF-treated neurons in culture, it was proposed that HSP70 levels were not up-regulated. The other explanation was that HSP70 levels increased, but contributed to the restriction of neurite outgrowth instead. This had been previously observed in PC12 cells where HSP70 inhibitor, KNK437, induces neurite outgrowth (Koike et al., 2006). In that model, HSP70 RNA levels are inhibited and neurite outgrowth is promoted, in the absence of stress stimulation. This process was blocked by the introduction of growth-signalling pathway inhibitors for ERK, p38 MAP kinases and glycogen synthase kinase 3β.

To assess whether HSP70 levels had increased in SCG neurons, HSP70 immunohistochemistry was performed on fixed young adult neurons in culture. After 21 hours, HSP70 levels did not change significantly in the presence of **AEG3482**. This result suggests that **AEG3482** could be working via a different mechanism, independent of HSP70, to restrict neurite outgrowth and that there was no effect on survival because HSP70 was not up-regulated. The other possibility is that HSP70 levels were elevated but this was not visualised.

Even though there was no significant increase in HSP70 levels of aged SCG during immunohistochemical studies, there was increased variability. This was observed in the standard error of HSP70 staining intensities, in NGF and AEG3482-treated young adult SCG cultured neurons, which was extremely high when normalised against control. This could be attributed to the different sub-populations of SCG neurons present in cell culture, which project to different targets, e.g. iris and cerebral vessels, and exhibit varying degrees of vulnerability in ageing.

Western blot data further demonstrated that levels of HSP70 were not found to significantly change between young adult and aged mice in sympathetic neurons. However, it is important to note that this data also includes protein expression in non-neuronal cells; HSP70 is also present in glia. It should also be taken into account that the HSP70 subfamily of HSP's have at least seven members in the mouse, including both constitutive and inducible forms (Esser et al., 2004). HSP's contain highly conserved N-terminal ATP-ase and C-terminal protein binding domains, so some cross-reactivity with the antibody may have occurred.

To explore the effects of JNK inhibition further, isoform-specific inhibitors were administered in culture to determine whether the survival and growth of NGF-treated SCG neurons is dependent on one particular JNK isoform or a combination of the three isoforms JNK1, 2 and 3. **JNK inhibitor VIII** and **JNK inhibitor IX** similarly showed no effect on neuronal survival of NGF-treated young adult and aged cultured SCG neurons but did inhibit neurite outgrowth. **JNK inhibitor IX** was highly effective at reducing neurite outgrowth in cultured SCG neurons and completely abolished neuritogenesis at a concentration of 10μM. In fact, **JNK inhibitor IX** and **AEG3483** exhibit similar responses when it comes to the neuronal survival in aged SCG neurons and the effects on glial processes (**see next section**), although they work through different modes of inhibition.

As **JNK inhibitor IX** inhibits JNK2 and JNK3 and **JNK inhibitor VIII** inhibits JNKI and JNK2 more effectively than JNK3, the results are consistent with JNK3 having a role in the neurite outgrowth of young adult and aged cultured SCG neurons. In practice, it may be difficult to assign certain function to a single JNK isoform due to the nature of the inhibitors used. Therefore, further experimentation with inhibitors that display added specificity is required to fully conclude this. JNK-specific siRNAs or organelle-specific targeting might also provide a way to observe isoform-specific functions in aged cell culture models, which is further deliberated in the **General Discussion(4.1.8)**.

3.2 The effect of JNK inhibition on growth and survival of ageing peripheral glia

3.2.1 Introduction

Knowledge of the role of glia in the nervous system has improved substantially in the last two decades and they are now accepted as key players in practically all aspects of nervous system function. Originally considered to have a limited role in supporting neurons, it has become clear that, without fully functioning glia, vital processes including the regeneration of neuronal networks, during atrophy and after injury, would be severely impaired.

3.2.1.1 Glia in the sympathetic system

Schwann cells and satellite glial cells are the non-neuronal glial cells found in sympathetic ganglia, and the immunoreactivity of glial cell markers in mouse and rat SCG has been previously described (Jessen and Mirsky 1984; Jessen et al., 1984; Schachner et al., 1984; Hall and Landis 1992; Okano et al., 2005). There is a difference in the expression of differentiated and non-differentiated Schwann cells; Glial fibrillary acidic protein (GFAP) is widely expressed *in situ* by non-myelin forming glial cells but not by myelin-forming Schwann cells, including those of sympathetic, nerves and ganglia from adult rats (Jessen and Mirsky 1984; Jessen et al., 1984; Hanani 2010).

While Schwann cells are the most familiar non-neuronal cells in sympathetic glia, satellite glial cells are the most common (Hanani 2010). Information gathered by the de Almeida-Leite and Arantes (2010) group produced the first report of mouse SCG glial cells from neonatal mice individually cultured and phenotypically characterised in pure primary cultures. In their study, a few glial cells were stained with S-100 (Schwann cell marker) and all with GFAP. Glia were described to have a flat and irregular shape, two or three long cytoplasm processes, and round, oval or elongated nuclei, with a regular outline consistent with previous studies in human and rat tissues (White 1889; Chiba and Williams 1975; Madariaga-Domich and Taxi 1986; Lein et al., 2002). It should be noted that not all studies agree on the composition of different types of glial cells in SCG. Perez-Gonzalez et al. (2008), found whole glial populations stained for S-100B in microcultures of SCG from neonatal rats.

Earlier in this study, GFAP immunohistochemistry was used to confirm the differentiation between neurons and glia (**Materials & Methods; Fig. 2.5**). Following the procedure of SCG dissociation and plating, the whole population of glia cultured from SCG of aged mice was weakly immunoreactive for GFAP. For the purpose of this study, there will be no attempt to distinguish between sub-types, however, current research into this field will be reviewed.

Neurons are individually enclosed by a tight envelope of satellite glial cells, which are distinct from Schwann cells in their association with neurons. Satellite glial cells are usually connected to a single neuron and wrap themselves around the soma to form a structural and functional unit, while Schwann cells are associated with several neuronal axons, forming a Remak bundle (Pannese et al., 1994; Campana 2007). Like satellite glial cells, Schwann cells are laminar structures but are usually elongated (approx 20–400 μm) (Pannese et al., 1994).

While there are plenty of studies on Schwann cells, ongoing research aims to fully determine the physiological role of satellite glial cells. So far they have been found to play a variety of roles, including control over the microenvironment of sympathetic ganglia, supply of nutrients to the surrounding neurons and also to act as protective, cushioning cells.

The maintenance of the characteristic phenotype of SCG neurons in culture have been found to be influenced by glial cells e.g. neurons in sympathetic ganglia form dendrites when grown in culture, but these are only extended when laminin-positive glia are present (Tropea et al., 1988). Tropea et al., (1988), indicated that co-culture with Schwann cells allowed sympathetic neurons to form a dendritic arbor comparable in size to that observed *in situ*, and that this cell-cell interaction was relatively specific. However a high concentration of non-neuronal cells were required to observe this effect. Findings by Perez-Gonzalez et al. (2008), have since demonstrated that removing non-neuronal cells from a culture system had no effect on neurite outgrowth in neonatal rat SCG. Similar results were observed in compartmental cell cultures of neonatal SCG neurons from neonatal mice, where glia were removed from the system (Howard et al., 2013).

3.2.1.2 Glial responses in ageing repair and regeneration

Pathological changes in sympathetic ganglia following nerve injury have been previously investigated (Kreutzberg 1995) and studies show morphological and physiological changes in neurons after axotomy of the postganglionic fibres e.g. retraction of synaptic contacts (Matthews and Nelson 1975). In order to reconnect these neuronal networks, the PNS undergoes a four-step regeneration process as described in the **Introduction (1.4)**, of which glia play an important role.

In ageing, it has been proposed that the decline in nerve regeneration after injury is brought about by changes in neuronal, axonal, Schwann cell and macrophage responses (Verdu et al., 2000). After injury, Wallerian degeneration in peripheral axons is delayed in aged animals (Tanaka 1991), which is believed to be brought about by a fading reactivity of non-neuronal cells (Verdu et al., 2000). Much larger myelin remnants accumulate in macrophages compared with young animals suggesting slower processing times (Tanaka et al., 1992). The interaction between Schwann cells and regenerative axons is also delayed, combined with a reduced concentration of trophic factors secreted by reactive Schwann cells in older subjects (Komiyama and Suzuki 1992). In turn, this may contribute to a reduction in the rate of axonal regeneration and the density of regenerating axons. Terminal and collateral sprouting of regenerated fibres is also affected, further restricting the success rate for target reinnervation and functional restoration. Importantly, the capabilities for axonal regeneration and reinnervation are still maintained throughout life, but are simply delayed and less effective.

The role of Schwann cells following injury has already been discussed (**Introduction 1.4, 1.5**) but satellite glial cells also have the ability to respond to various kinds of injury or disease, and their close contact to neurons allows small amounts of substances released to influence neuronal function (Liutkiene et al., 2007; Griffin and Thompson 2008; Hanani 2010), and promote *in vitro* neurite growth (Lein et al., 2002). Like Schwann cells, satellite glial cells are highly motile; (Chamley et al., 1972), suggesting that they can change their position and morphology, something that is essential for repair following peripheral damage and during phagocytosis.

Nerve injury also prompts 'synaptic stripping' (Kreutzberg 1995), a process where neurons lose their synaptic inputs and become physically separated from them after

axotomy. While microglia facilitate synaptic stripping in the CNS (Schiefer et al., 1999), macrophages implement this in SCG (Schreiber et al., 1995). The involvement of satellite glial cells is still unclear but detached presynaptic elements were found enveloped by lamellae of satellite glial cell cytoplasm (Matthews and Nelson 1975) thus implicating satellite glial cells in this process.

Mechanical insult to the peripheral nerve initiates a cascade of molecular events in the distal nerve stump that promotes myelin degeneration followed by dedifferentiation and proliferation of the Schwann cells. In adult animals, when the nerve is damaged, myelinating and non-myelinating Schwann cells have the ability to revert back to a non-differentiated, proliferative phenotype. It has recently been suggested that this injury-induced cell plasticity facilitates a transdifferentiation that generates a specialised repair cell, also termed a Büngner cell, which can be distinguished from the Schwann cells found in the developing nerve. These repair cells guide regrowth of the injured axons and eventually remyelinate them to allow functional recovery of the damaged nerve (Arthur-Farraj et al., 2012).

Schwann cells are also involved in other repair processes such as the breakdown of the blood-nerve barrier and the recruitment of macrophages to the site of injury to clear myelin debris. Further studies are necessary to completely understand the molecular mechanisms that control the regenerative properties of the Schwann cell. What is clear is that adult Schwann cell plasticity is regulated by a complex array of signalling pathways and transcription factors in response to injury. Among these are JNKs, as well as ERKs and p38 MAPK, which are involved in mediating the injury signal that initiates the Schwann cell de-differentiation process. These MAPKs act as negative regulators of Schwann cell differentiation and myelination, thus facilitating the transition toward the immature phenotype (Myers et al., 2003; Harrisingh et al., 2004; Ogata et al., 2004; Zrouri et al., 2004; Parkinson et al., 2008; Syed et al., 2010; Yang et al., 2012). Despite links with JNK (Parkinson et al., 2004; Monje et al., 2010), more focus has been placed on the ERK/p38 MAPK signalling pathways (Harrisingh et al., 2004; Guertin et al., 2005; Napoli et al., 2012; Yang et al., 2012).

3.2.1.3 Glial responses further downstream

While c-Jun has been shown to play a significant role in the regeneration of neurons (Raivich et al., 2004; Ruff et al., 2012), it has also been assessed in Schwann cells during the functional repair of peripheral nerves after injury (Jessen and Mirsky 2008; Arthur-Farraj et al., 2012). A downstream target of JNK, c-Jun is up-regulated after injury in both myelinating and non-myelinating Schwann cells that are removed from axonal contact of sensory neurons (Parkinson et al., 2008). Satellite glial cells have also been implicated in pathological changes following injury to SCG and observations indicate induced expression of c-Jun, Jun B and Jun D (Koistinaho et al., 1993).

Conditional deletion of c-Jun in Schwann cells does not appear to affect development or myelination of peripheral nerves. However, in adult nerves following injury, Schwann cells lacking c-Jun failed to down-regulate myelin proteins and take on the repair cell state. This distinct lack of repair in c-Jun-null nerves suggested that the formation of Büngner cells from the dedifferentiation of Schwann cells is dependent on c-Jun (Arthur-Farraj et al., 2012). Studies have since shown that sciatic nerve injury induced c-Jun activation is JNK-independent in adult Schwann cells (Blom et al., 2014), but this is yet to be explored in sympathetic glia.

3.2.1.4 Growth factor signalling

Ultimately the function of Schwann cells is specifically to produce both the environment and neurotrophic support necessary for effective axonal regeneration, and repair of the adult nerve. The actions of NGF and other neurotrophins on sympathetic neurons are well elucidated but the role of satellite glial cells and non-myelinating Schwann cells in neurotrophic signalling and, conversely, the trophic effect on glia, has received less attention.

Previous studies reveal that Schwann cells are NGF-responsive (Taniuchi et al., 1988) but they also have the ability to synthesise and secrete a wide range of growth factors, NGF being one of them (Assouline et al., 1987; Heumann et al., 1987; Bampton and Taylor 2005). NGF levels are increased in Schwann cells post-axotomy (Assouline et al., 1987; Bandtlow et al., 1987; Matsuoka et al., 1991; Weidner et al., 1999) and it has been suggested that injury acts as a trigger to induce neurotrophin production

(Taniuchi et al., 1988; Matsuoka et al., 1991). Immunohistochemistry and *in situ* hybridization by Wetmore and Olson (1995) revealed that satellite glial cells in adult rat SCG contain BDNF mRNA and TrkB transcripts but not TrkA and TrkC suggesting that NGF production and responsiveness is limited to Schwann cells in that environment.

3.2.2 Aims

In current research, much consideration has been given to the importance of JNK in neurodegeneration and age-related diseases. However, there is still much to be learnt about the physiological responses of glia in aged sympathetic ganglia.

In the first section of this study, experiments also demonstrated an impact on the growth of glial extensions, as well as glial proliferation, in response to JNK inhibition. Therefore, the hypothesis is that JNK inhibition has an important role to play in the differentiation of young adult and aged SCG glia too.

There may also be an indirect effect of glia on neuronal responses in young adult and aged SCG cultures. In this section, the effect of JNK of young adult and aged glial phenotype will be assessed, including their relationship with neurons.

3.2.3 Results

3.2.3.1 Effect of JNK inhibition on glial proliferation and process outgrowth

Unlike neurons, glial cells possess the ability to divide post-development, and often proliferate when explanted in culture. As cultures in the first section were treated with AraC (**see Methods, 2.2.4**) to suppress any glial proliferation, it was possible that the phenotypes of the glia were altered and the effects of JNK inhibition were masked. In order to clarify this, the survival and growth of glia in the presence of JNK inhibitors was assessed in AraC-free medium.

Without AraC in control SCG cultures of young adult and aged dissociated neuronal and non-neuronal cells, glia proliferated extensively, and grew long processes by day 2 (**see Methods**). On Day 1 of culture, glial cells are yet to form glial extensions so photographs were taken to observe the effect of different JNK inhibitors on Day 3 (**Fig 3.9**).

AEG3482 and **JNK Inhibitor II** completely restricted the growth of glial extensions and arrested glial proliferation. **JNK Inhibitor III** was less effective at restricting glial extension than **JNK inhibitor II. JNK inhibitor VIII** (inhibits JNK 1+2 more effectively that JNK 3) also showed some restriction of growth. However, **JNK inhibitor IX**, ATP competitive inhibitor of JNK2 and JNK3, completely abolished the formation of glial extensions. By Day 3, the cultures appeared to possess a lot of debris, with glial processes breaking off and the glial cell body shrivelling up. It is not possible to determine whether glial cells are alive by morphological criteria, therefore, to elucidate whether JNK inhibition purely restricts growth or kills glial cells, a Live/Dead assay was performed on Day 2 of culture. Results clearly showed that there was no effect of **JNK inhibitor IX** on glial survival **(Figure 3.9, G)** and what was being observed was a clear restriction in the formation of glial processes.

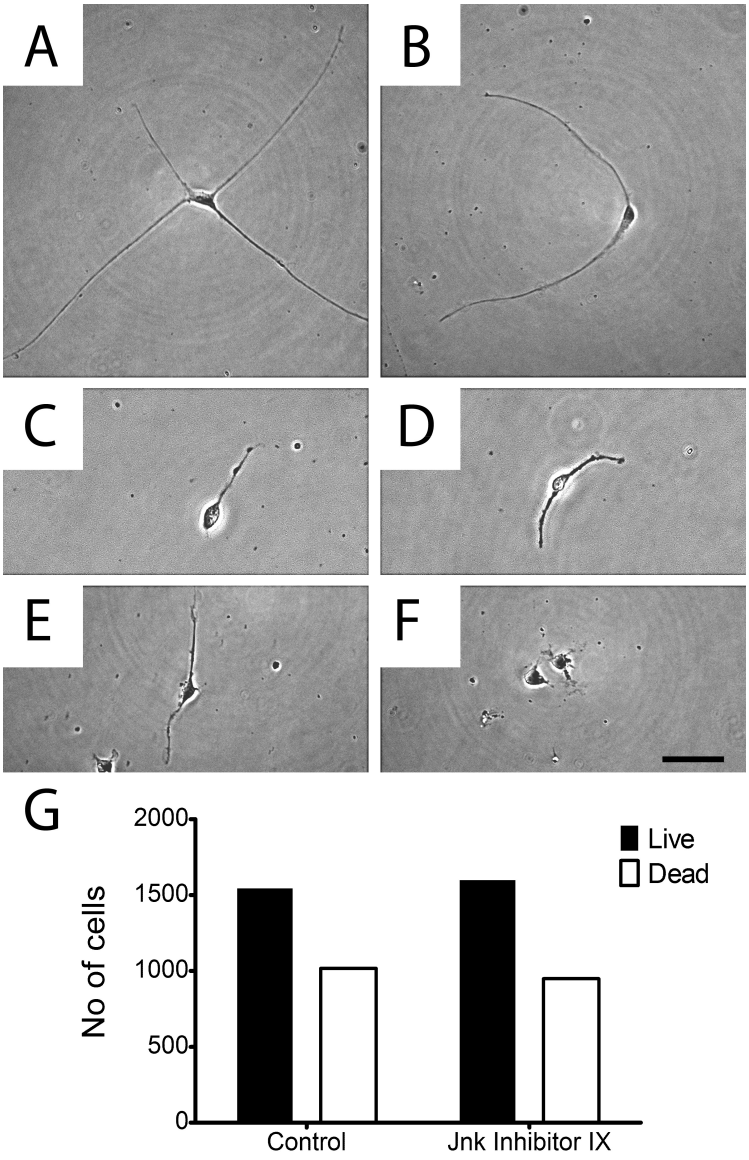


Figure 3.9: Representative phase contrast images of NGF-treated glial cells in aged SCG cultures after the treatment of various JNK inhibitors for 72 hrs.

All cultures were NGF (0.4nM) treated and the JNK inhibitors were administered at 10μM, AEG3482 (40μM). **(A)** NGF control, **(B)** JNK inhibitor III, **(C)** AEG3482, **(D)** JNK inhibitor II, **(E)** JNK inhibitor VIII and **(F)** JNK inhibitor IX. Scale bar = 100μM **(G)** Live-dead assay demonstrated that the survival of JNK inhibitor IX-treated glia is not significantly different from that of NGF control, despite the dramatic effect on growth and the unhealthy appearance of the cells ($p=0.91$). Data is representative of three experiments.

3.2.3.2 Glial impact on neurons

Glial cells possess the ability to promote trophic effects in neuronal cells through a local supply of NGF (Assouline et al., 1987) , although neurite outgrowth is not solely dependent on the NGF secreted by glia. It is difficult in these experiments to evaluate the difference between young adult and aged cultured neurons because each culture requires different preparation. However, similar trends were observed in the effect of various JNK inhibitors on the NGF-dependent growth of neurites and glial processes **(Table 3.1**) in adult and aged SCG cultures, with the addition or absence of AraC. While JNK inhibition may not be signalling through the same pathway in neurons and glia, similar effects on growth are observed in both cell populations.

Table 3.1: Summary of the effects of JNK-inhibiting compounds on the growth of NGF-treated SCG glia and neurons from young adult and aged mice in the absence of AraC.

Inhibitor	Mode of action	Growth of glial processes	Growth of neurites
JNKi II	ATP competitive inhibition of JNK	Substantial restriction	Substantial restriction
JNKi III	Cell permeable peptide disrupts cJun/JNK	Some restriction	Some restriction
JNKi VIII	ATP competitive inhibition of JNK 1+2	Some restriction	Some restriction
JNKi IX	ATP competitive inhibition of JNK 2+3	Completely restricted	Completely restricted
AEG3482	Bind HSP90, which indirectly increases HSP70, which inhibits JNK	Substantial restriction	Substantial restriction

3.2.4 Discussion

The growth and proliferation of glia was restricted by JNK inhibition in cell cultures of aged mouse SCG. Under these conditions, **AEG3482**, **pan-JNK inhibitor II** and **JNK inhibitor IX** had the strongest inhibitory effect whereby glial proliferation was arrested and the growth of glial extensions was severely restrained.

There was some restriction of **JNK inhibitor III** (**Fig 3.9**) on glial processes in culture, while the impact of **JNK inhibitor VIII** was slightly more pronounced. Interestingly, within cultures treated with **JNK inhibitor IX**, glia appeared shrunken, with complete elimination in the extension of glial processes. In order to determine whether this appearance signified cell death, or simply a considerable impact on growth without cell death, a Live/Dead assay was performed. Results confirmed that, while there was a radical change to the glial phenotype, the cells retained both intact membranes and intracellular esterase activity, suggesting that **JNK inhibitor IX** did not affect glial survival. As **JNK inhibitor IX** (ATP competitive inhibitor of JNK2 and JNK3) is much more effective at restricting glial process and proliferation than **JNK Inhibitor VIII** (inhibits JNK 1+2 more effectively that JNK 3), these observations suggest that, as with neurons, JNK3 is likely to be particularly important for the growth of glial cells.

These results may have therapeutic implications in the use of JNK inhibitors for neurodegeneration and neuronal injuries acquired at any stage of life. It has been previously discussed that the JNK signalling pathway could be a potential therapeutic target in peripheral nerve injury and repair due to activation through a wide range of cellular stresses, as well as inflammatory mediators, which are also active in nerve injury. While emphasis has generally been consigned to the targeting of JNK signalling in neurons, there may also be benefits to inhibiting JNK in glia under harmful conditions. In particular, gliosis in the brain is an increasing problem, whereby a proliferation of glia following brain injury can be damaging to the surrounding neurons (Robel et al., 2011). Similarly, glial scar formation due to reactive astrogliosis following spinal cord injury forms an impenetrable barrier for regenerating axons (Yuan and He 2013). It may be that treatment with JNK inhibitors are useful for an acute period of time to inhibit the formation of additional glia, without affecting the survival of existing glia and nearby neurons.

The time it takes to facilitate nerve repair is a significant challenge when it comes to ageing. The complex signal transduction pathways initiated in cells following peripheral nerve injury, and the interactions between neurons and glia are delayed in older subjects. Therefore, further studies would be required using extended time periods to make sure there is no long-term detriment to regeneration and outgrowth.

Further downstream, it has been found that sciatic nerve injury-induced c-Jun up-regulation, which is necessary for the formation of Bunger cells, is JNK-independent (Blom et al., 2014). Induced expression of c-Jun, Jun B and Jun D has also been observed in satellite glial cells following injury to SCG (Koistinaho et al., 1993), however it is yet to be determined whether this is JNK-independent. Therefore, it would be relevant to assess c-Jun and other downstream targets affected by JNK inhibition, and the restrictive activity controlling glial process outgrowth and proliferation. Some inhibition of glial process outgrowth was also observed in cultures treated with **pan-JNK Inhibitor III**, which is a cell permeable peptide that specifically disrupts the JNK/c-Jun interaction. Therefore, JNK/c-Jun is likely to be involved to some extent in the process of young adult and aged SCG glial proliferation but further studies are required to confirm this.

While **pan-JNK inhibitor II** works by ATP competitive inhibition of JNK, it is not known through which mechanism **AEG3482** inhibits growth. Interestingly, work by Hou et al. (1998) found an up-regulation of HSP70 in satellite glial cells, as well as neurons, in rat SCG after carotid nerve axotomy, which suggests a role for HSP70 in glia as well. Results in the previous section show that HSP70 levels are unaffected in young adult and aged neurons after the administration of **AEG3482**, which suggests that JNK-inhibition induced restriction of neurite outgrowth is perhaps occurring through a different mechanism. Further studies are required to elucidate whether this is also true of glia.

When SCGs are dissociated and cultured, neurons are damaged, so it is likely they are in a state of repair. In the periphery, regeneration of neuronal networks is facilitated with the support of Schwann cells, satellite glial cells and macrophages. However, in culture conditions, as those described above, which have a low plating density, glial cells are sometimes far from neurons. In addition, neurons attached to glial cell bodies were not scored. Due to the nature of the methodological process in culturing neurons, it is also possible that some glia are removed via differential sedimentation.

Therefore neurons received little or no glial support in this culture model. This is further supported by the lack of an effect of AraC (inhibits glial proliferation and process outgrowth) on the survival and growth of neurons on Day 2 of culture (**Materials and Methods, 2.2.4**).

Going forwards, it would also be of interest to fully characterise the population and composition of specific glia post-injury in adult SCG and during ageing. As satellite glial cells and Schwann cells display different characteristics and possess different biological functions, it would be relevant to understand the regulatory processes involved. To help differentiate between different classes of glia, Taveggia et al. (2005), proposed treating all Schwann cells as non-myelinating unless they possess threshold levels of neuregulin-1 type III (NRG1), a member of the epidermal growth factor (EGF) family. Satellite glial cells, however, do not require neuregulin for normal developments in sympathetic ganglia (Jessen and Mirsky 2005) so this provides a method of distinguishing between the two glial sub-types.

Having filtered through the literature, the information on the influence of sympathetic glia on neuronal behaviour is still sparse and will require much more attention to fully decipher their relationship in ageing and following injury or insult.

3.3 Is proNGF signalling JNK dependent?

3.3.1 Introduction

For many years, the unavailability of NGF was thought to be responsible for a neurotoxic effect in neurons in ageing (**see Introduction**). However, since the turn of the century, reports have indicated that proNGF mediates this effect and has an essential role in neurodegeneration. Furthermore, proNGF has also been implicated in the neurite outgrowth of subpopulations of NGF-responsive young adult and aged neurons and may also be important for neuronal repair after injury (Al-Shawi et al., 2008).

Throughout life, the amount of proNGF is dynamically regulated and levels have been reported to increase in ageing (Nykjaer et al., 2004; Al-Shawi et al., 2008), disease (Pedraza et al., 2005; Chen et al., 2008; Stoica et al., 2008; Belrose et al., 2014) and after injury (Harrington et al., 2004). Fahnestock et al. (2001) found proNGF to be the predominant form of NGF protein in adult mouse, rat, and human brain tissue, whereas very limited levels of mature NGF (mNGF) were detected; lack of mNGF may be a result of being rapidly transported after being processed. The levels of proNGF continue to increase in SCG of one-year old mice, which show a higher concentration compared with 7-week old adult animals (Jansen et al., 2007). This observation also ties in with reports of a 50-fold increase of proNGF levels in sympathetic ganglia of two-year old rats (Bierl and Isaacson 2007).

Interestingly, increased proNGF levels in aged SCGs were not paralleled by a similar rise in mRNA concentrations, as determined by RT-PCR, suggesting that post-transcriptional mechanisms affect neurotrophin production and accumulation in the tissue (Hasan et al., 2003; Jansen et al., 2007). Work by Jansen et al. (2007) suggested that it was unlikely for retrograde transport of target-derived proteins to be responsible for accumulation of proNGF in SCGs because no increase in pro-neurotrophin production was evident in aged middle cerebral arteries, following analysis of Western blotting or RT-PCR. However, strong co-localisation was observed between proNGF and tyrosine hydroxylase, which defines sympathetic neurons using double immunostaining. Therefore, it was concluded that proNGF may originate from ageing SCG neurons themselves. Local production of proNGF was also confirmed by *in situ* hybridisation, where NGF mRNA transcripts were found in SCG neurons from 60-week old mice.

Developmental regulation of proNGF levels forms the basis for the differing responses of proNGF signalling. Unlike young adult neurons, NGF-responsive BFCN and SCG neurons from aged rodents are vulnerable to proNGF-mediated cell death (Jansen et al., 2007; Al-Shawi et al., 2008). As well as an exposure to increased levels of proNGF *in vivo* (Bierl and Isaacson 2007; Al-Shawi et al., 2008), aged neurons express higher levels of sortilin (Al-Shawi et al., 2008), which acts to enhance binding of proNGF to $p75^{NTR}$ (Nykjaer et al., 2004). Therefore execution of this neurotoxic signalling function may be determined by both the availability of proneurotrophins, and cell surface receptors (Fahnestock et al., 2004; Al-Shawi et al., 2008; Masoudi et al., 2009). This has been further supported in PC12 cells where increased vulnerability to proNGF-mediated cell death is observed under conditions in which the ratio of $p75^{NTR}$ relative to TrKA is increased (Masoudi et al., 2009).

The dependence on neurotrophin availability has also been observed in subpopulations of SCG neurons, which exhibit different degrees of vulnerability during ageing. Rat SCG neurons projecting to cerebral blood vessels (CV) are significantly reduced with a cell loss of 15% by mid-age with further losses by old age, while iris-projecting neurons only suffer significant losses by old age (Al-Shawi et al., 2008). Interestingly, Al-Shawi et al. (2008) found twofold lower levels of proNGF in the iris compared with the CV at mid-age. Therefore, the distribution of proNGF in the nervous system has become a vital part of research, especially as earlier antibody studies failed to distinguish between neurotrophins and their precursors.

Differential regulation of proNGF signalling may be achieved through the existence of several proNGF forms (**Introduction 1.3**). Two different transcript products (Ullrich 1983, Edwards 1986), accompanied by several potential targeted sites for convertases and glycosidase can create different proNGF forms, and can vary from one tissue to another. Pedraza et al. (2005) discovered four different immunoreactive bands corresponding to proNGF in human brain tissue affected by AD alone (26, 32, 37 and 53kDa). Densitometry analysis further presented differences in the increase of different proforms, at different stages of AD, in different sections of brain. In entorhinal cortex, the 53kDa band increased and, in frontal cortex, the same proform increased along with 37 and 32kDa forms of proNGF. Three of these proforms induced apoptotic cell death (53, 32 and 26kDa) when pooled in $p75^{NTR}$:SCG primary neurons cultures.

For proNGF-mediated growth signalling, some suggest an initial requirement for proNGF cleavage to mNGF, which subsequently binds and signals via TrkA, as shown in PC12 cells (Boutilier et al., 2008) and in the presence of glia (Kalous et al., 2012). In adult sensory neurons (DRG), Kalous et al. (2012) proposed that the proNGF-mediated neurotrophic response is indirectly mediated by NGF and TrkA, through sortilin- and $p75^{NTR}$-dependent cleavage of proNGF, to mNGF, in peri-somatic glia. The potential ability of a targeted local supply of mNGF supplied by glial cells may provide an important regulatory action to promote trophic outcomes, as opposed to cell death, under conditions where regeneration or sprouting is required. Interestingly, immunocytochemistry in sections of young adult and aged SCG from old mice suggest that sortilin is not present in glia from sympathetic neurons (Al-Shawi et al., 2008). Therefore, the outcome of proNGF signalling in SCG may be partly regulated by cleavage through pro-protein convertases e.g. furin, proteases e.g. MMP-7, and not glia. In any case, proNGF has still been found to have neurotrophic effects without any cleavage at all. Howard et al. (2013) demonstrated that in the absence of surrounding glia, using compartmentalised chambers, cleavage-resistant proNGF can still promote neuritogenesis of developing cultured sympathetic neurons.

In experimental studies, different forms of proNGF have also been developed over time, from wild type to cleavage-resistant mutations, of which binding characteristics have been evaluated (Clewes et al., 2008; Masoudi et al., 2009). This, coupled with the methodological differences employed in different studies, may contribute to the differing cellular responses of proNGF (**Introduction 1.3**).

For example, Fahnestock et al. (2004) used a cleavage-resistant proNGF (34kDa), produced in Sf9 insect cells, which disrupts the tetrabasic cleavage site (R-1G). A high density of SCG neurons were cultured (20,000 neurons per 35 mm dish), and an increase in the proportion of growing neurons, with enhanced survival, was observed through binding TrkA, when treated with proNGF. Similar results were obtained by Howard et al. (2013), using the same cleavage-resistant proNGF. In contrast, the cleavage-resistant proNGF administered by (Lee et al., 2001), which has different amino acid substitutions (−2, −1, 118 and 119. RR to AA), promoted apoptosis and had negligible interactions with TrkA, thus preventing neurite growth from SCG neurons (Park et al., 2001). However, Masoudi et al. (2009) discovered proNGF

enhanced the survival of SCG neurons, rather than promoting apoptosis, and increased the number of neurons with neurite outgrowth, with the same cleavage-resistant proNGF as Lee et al. (2001). Masoudi et al. (2009) obtained similar results when using an additional cleavage-resistant proNGF with multiple amino acid substitutions (−73 and −72, RR to AA; −43 and −42, KKRR to KAAR; and −2 and −1, KR to AA) (Pagadala et al., 2006) and another with two different substitutions (−1 and +1 RS to AA). Importantly, SCG neurons were also plated at an exceptionally high density in these studies.

Clewes et al. (2008) used wild-type proNGF, and a human recombinant furin-resistant proNGF (M-proNGF) R121A, S122A (both 24.8kDa), produced from *E. coli* and refolded, devoid of extraneous purification tags. M-proNGF was bound to HEKTrkA at the same affinity as wild-type proNGF. Both M-proNGF and mNGF-induced phosphorylation of TrkA and ERK1/2 signalling. In the Howard et al. (2013) study, two different forms of cleavage-resistant proNGF and native proNGF elicited neurite growth of the same magnitude and neuronal specificity. For studies in aged SCG neurons, Al-Shawi et al. (2008) administered a wild-type proNGF in culture, and observed neurite outgrowth in cultured young adult and aged SCG neuron from aged rats. Interestingly, no significant cell-death of young adult SCG neurons was observed in this study but there was neurotoxicity in aged SCG; so different methodology doesn't necessarily explain age dependent cell-death.

Comprehensive studies, *in vitro,* have shown that cleavage-resistant proNGF can promote neurite outgrowth in populations of NGF-responsive neurons but can also mediate a neurotoxic effect in aged neurons. Overall, it may be that in ageing, proNGF-mediated regulation of survival and growth *in vivo* is determined by the availability of intracellular and extracellular cleavage proteins which cleave proNGF to mNGF, but also the local supply of proNGF, combined with the expression of receptor complements e.g. sortilin and $p75^{NTR}$ or TrkA. Regulation of downstream effectors may also be important.

3.3.1.1 Role of NRAGE in aged neurons

NRAGE, an 86kDa cytoplasmic protein, was first identified by Salehi et al. (2000), to directly interact with $p75^{NTR}$ and facilitate apoptosis of NGF-responsive neurons in PC12 cells (Salehi et al., 2002). NRAGE contains a ~200-amino acid MAGE homology domain (MHD) (Barker and Salehi 2002), which binds to the juxtamembrane domain of $p75^{NTR}$ *in vitro* and *in vivo*. It acts by blocking the physical association of $p75^{NTR}$ with TrkA and appears to compete with TrkA for the same site on $p75^{NTR}$. NRAGE over-expression facilitates cell cycle arrest and $p75^{NTR}$-dependent apoptosis within PC12 cells. In contrast, TrkA overexpression abolishes NRAGE-mediated death, suggesting $p75^{NTR}$ forms independent complexes either with NRAGE or with TrkA. NRAGE expression was also necessary for $p75^{NTR}$-dependent apoptosis of MAH cells and this was blocked by TrkA expression (Salehi et al., 2000).

$p75^{NTR}$/NRAGE dependent apoptosis involves the JNK pathway, and subsequent cytosolic accumulation of cytochrome *c*, activation of Caspases-3, 9 and 7, and caspase-dependent cell death in NGF-responsive neurons (Salehi et al., 2002; Bhakar et al., 2003; Salehi et al., 2006). Blocking downstream targets; JNK and c-Jun, by overexpression of the JNK-binding domain of JIP1 or dominant-negative c-Jun, arrests NRAGE-mediated caspase activation and NRAGE-induced apoptosis in PC12 cells (Salehi et al., 2002). *In vivo* studies on NRAGE KO mice, and studies on primary sympathetic neurons derived from these mice, also support a role for NRAGE in $p75^{NTR}$ -mediated cell death (Bertrand et al., 2008).

NRAGE is also involved in neuritogenesis, although the exact role is yet to be confirmed due to a certain degree of variability. In PC12 cells, which differentiate in response to NGF, NRAGE was found to act as a negative regulator of neurite outgrowth by activation of the TrkA-ERK signalling pathway (Feng et al., 2010). However, growth-promoting effects have been reported by Reddy et al. (2010), while in NRAGE KO mice, neuronal growth was found to be normal (Bertrand et al., 2008). Again, these patterns have only been observed in PC12 cells and development, so it is relevant to observe the impact in aged neurons.

3.3.2 Aims

Apoptosis in NGF-dependent neurons is facilitated by $p75^{NTR}$ and its cytosolic interactor, NRAGE, but attenuated by JNK inhibition. Pan-neurotrophin receptor, $p75^{NTR}$, is also required to elicit proNGF-mediated neurotoxic effects in ageing, therefore it is hypothesised that NRAGE and JNK are also involved in this signalling response.

Similarly, proNGF has been shown to promote neurotrophic effects in aged sympathetic cultures although the molecular pathway through which it acts is still unclear.

This section aims to observe whether the proNGF-mediated cell death and growth promoting activities are JNK-dependent, by administering JNK inhibitors in proNGF-treated SCG neuron cultures from aged mice. NRAGE siRNA knockdown in the same cultures will also seek to establish whether $p75^{NTR}$/NRAGE is involved in the proNGF- signalling response in aged neurons.

3.3.3 Results

SCG neurons from aged mice were cultured as normal then subjected to cleavage-resistant proNGF (Alomone Labs) treatment. Young adult neurons were not assessed because proNGF was not neurotoxic in whole populations of cultured SCG neurons from this age-group, as determined by Al-Shawi et al. (2008).

Following administration of 0.6nM proNGF, there was no significant effect in the survival of aged SCG neurons on Day 1 (**Fig 3.10**). In some systems, **NRAGE** activity has been identified to take 2-3 days activation (Salehi et al., 2000), so to observe whether there was a delayed response, cells were followed for three days. On Day 3, the survival of proNGF-treated neurons still remained insignificantly different from control-treated cultures.

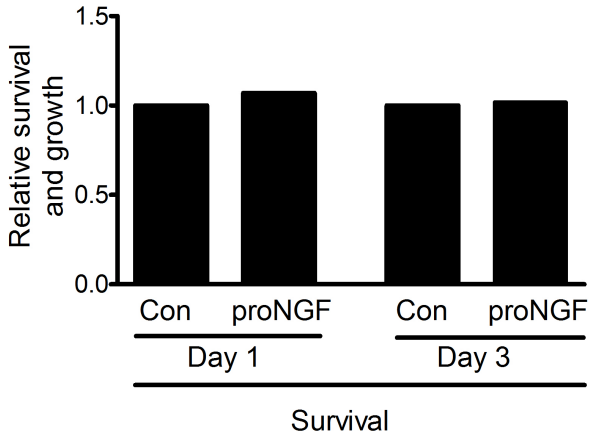


Figure 3.10: Survival of aged sympathetic neurons in pro-NGF treated cultures

(Day 1+3) proNGF (Alamone) treatment (0.6nM) did not have a significant effect on neuronal survival, relative to control (n=300).

To maximise any response, a higher concentration of proNGF (1.2mM) was administered, and survival and growth was monitored. Cells were followed for 3 days as before.

The effect on the survival of proNGF-treated aged SCG neurons was insignificant on Day 1 and Day 3. However, the proportion of growing neurons in proNGF-treated wells relative to control is increased by 4-fold in aged SCG cultures containing 1.2nM proNGF on Day 1 **(Fig 3.11)**; on Day 3, this ratio fell to 3-fold because of a delayed growth response in control cultures.

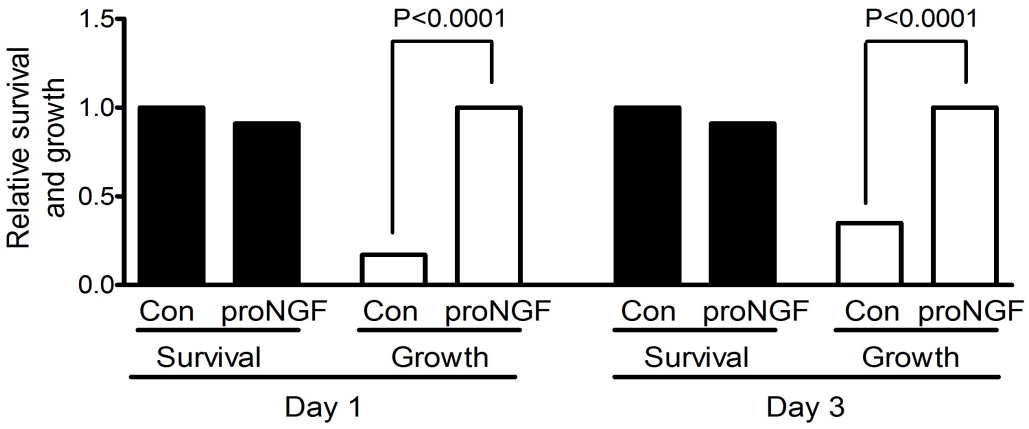


Figure 3.11: Survival and neurite outgrowth of aged sympathetic neurons in proNGF-treated cultures

(Day 1 and Day 3) proNGF (1.2 nM) treatment had no significant effect on survival but significantly increased the proportion of aged SCG neurons with neurite outgrowth, relative to control ($p<0.0001$; $n=300$).

3.3.3.1 proNGF-mediated toxicity in the absence of insulin

One difference between these experiments and those conducted by Al-Shawi et al. (2008), whereby a neurotoxic effect on aged SCG neurons was observed in proNGF-treated cultures, was the presence of insulin in serum-free culture media.

Previous work on the brain has demonstrated neuroprotective roles for insulin, a polypeptide hormone that serves as a growth factor amongst a host of other functions, such as regulating enzymatic pathways involved in energy storage, and synthesis of structural proteins. A role for insulin in central neurons following cerebral ischemia is to activate protein kinase B (PKB/Akt), which subsequently mediates anti-apoptotic actions of growth factors in cells (Downward 1998). In rat hippocampal neurons, activation of Akt1, via stress-induced ischemia, inhibited phosphorylation of JNK1/2, c-Jun, Bcl-2 and attenuated caspase-3 activation thus halting cell death (Hui et al., 2005).

Binding of insulin to the insulin receptor causes receptor auto-phosphorylation and exposure of phosphorylated residues that function as docking sites for insulin receptor substrate (IRS) and adaptor proteins (Shc, Gab1, APS). Phosphorylation of IRS-1 promotes the activation of the p85 regulatory subunit of PI3K, which subsequently modulates PKB/Akt (Gallagher et al., 2010). Previous studies in rat sympathetic neurons during early development demonstrated that overexpression of PI3K or its downstream effector Akt blocked cell death after NGF withdrawal, in spite of the fact that the c-Jun pathway was activated (Philpott et al., 1997).

To eliminate the effects of insulin on survival and growth, as previously (Al-Shawi et al., 2008), further experiments were carried out in insulin-free medium (neurobasal-A medium supplemented with insulin-free B27). Cells were followed for three days to observe whether there was a delayed or prolonged effect of proNGF.

In insulin-free medium, proNGF significantly reduced the survival of SCG neurons from aged mice on Day 1 and Day 3 (**Fig 3.12**). The percentage difference in survival of proNGF-treated cells, relative to control, was approximately 32% on Day 1 and 17% on Day 3, suggesting that proNGF-induced cell death is set into motion at an early

stage or that there is a larger population of dead neurons in control-treated wells, on Day 3, thus lowering the control population of surviving neurons.

In the same cultures, neurite outgrowth was substantially improved in the presence of proNGF. On Day 1, the proportion of growing neurons in proNGF-treated cultures was 4-fold higher than the control, and this proportion of growing neurons, relative to control, was maintained across the three days.

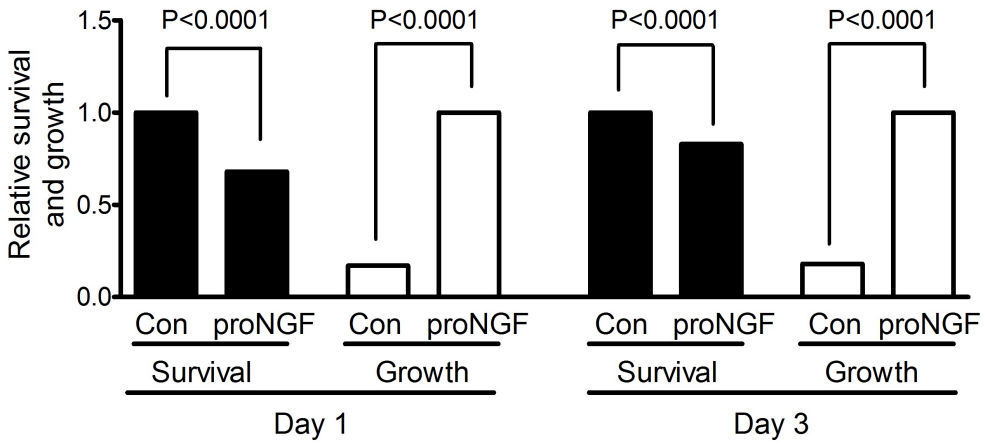


Figure 3.12: Survival and neurite outgrowth of proNGF-treated aged sympathetic neurons (without insulin)

(Day 1 and Day 3) proNGF treatment significantly reduced neuronal survival ($p<0.0001$) and increased the proportion of aged sympathetic neurons with neurite outgrowth, relative to control ($p<0.0001$; $n=900$).

Earlier in this study, experiments on aged mice revealed that **AEG3482** had no effect on the survival of SCG neurons, possibly because older neurons are not dependent on NGF for survival. Therefore, to identify whether JNK is involved in proNGF-mediated killing of aged neurons, **AEG3482** was administered to proNGF-treated cultures.

In cultured aged SCG neurons, proNGF-mediated cell death (approx. 30%) was inhibited in the presence of **AEG3482 (Fig. 3.13)**. In the same cultures, proNGF-mediated growth of SCG neurons was restricted by **AEG3482**. The proportion of growing neurons in culture compared to proNGF-treated cultures alone was reduced by 85% on Day 1. Altogether, these results imply that JNK is involved in proNGF-mediated signalling. On Day 3, the ratio of surviving neurons compared to proNGF control was increased in **AEG3482**-treated cultures, although neurite outgrowth was completely eliminated.

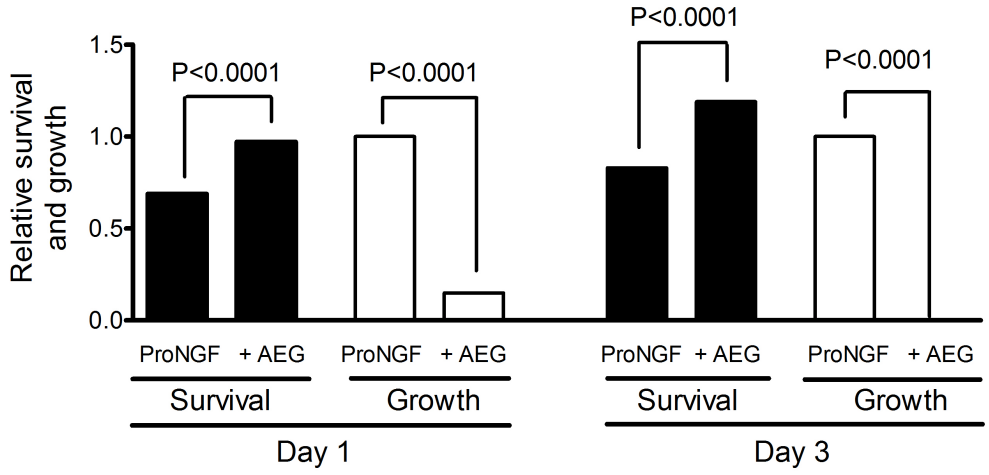


Figure 3.13: AEG3482 rescues aged SCG neurons from the neurotoxic effect of proNGF and reduces proNGF-induced neurite outgrowth

(Day 1) AEG3482 rescued neurons from proNGF-mediated cell-death ($p<0.0001$), and significantly decreased the proportion of proNGF-induced aged SCG neurons with neurite outgrowth ($p<0.0001$). (Day 3) AEG3482 rescued neurons from proNGF-mediated cell-death ($p<0.0001$), and eliminated any proNGF-induced neurite outgrowth of aged SCG neurons ($p<0.0001$) ($n= 900$).

3.3.3.2 NRAGE

AEG3482 rescues neonatal SCG neurons from NRAGE-mediated cell death through JNK, so further experiments were required to determine if the deleterious effects of proNGF on aged neurons are also mediated by NRAGE.

In these experiments, NRAGE knockdown was achieved by siRNA, which was administered in culture **(Materials & Methods 2.8)**. RNA oligos were coupled to Penetratin-1 prior to delivery. To confirm the efficacy of NRAGE knockdown, siRNA was administered in neonatal SCG cultures in the absence of NGF. Newly isolated sympathetic neurons, derived from rodents, die within 24 hours of plating in culture unless provided with NGF (Chun and Patterson 1977). Therefore, in the neonatal experiment, NGF was included within all media post-SCG excision. NGF-free medium was substituted in culture wells containing control siRNA and NRAGE siRNA.

Findings show that the survival of neonatal neurons was significantly better in the presence of NRAGE siRNA, following NGF withdrawal. As expected, siRNA knockdown of NRAGE prevented the death of neonatal SCG neurons by 13%, all relative to NGF control. In contrast, experiments on SCG neurons cultured from old mice demonstrated that NRAGE siRNA had no significant effect on neuronal survival on Day 1 (**Fig. 3.14**).

Data also revealed that NRAGE siRNA had no effect on the neurite outgrowth of aged SCG neurons on Day 1 and that Penetratin-1 had no significant effect on the survival and growth of NGF-treated SCG neuron cultures (results not shown).

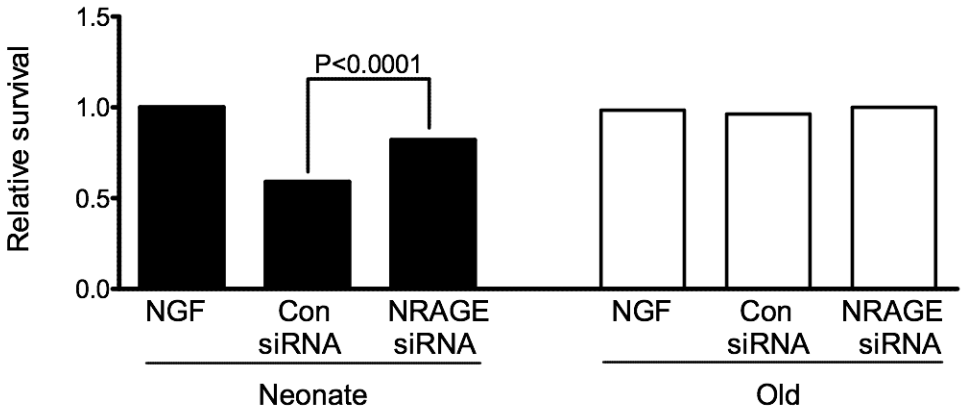


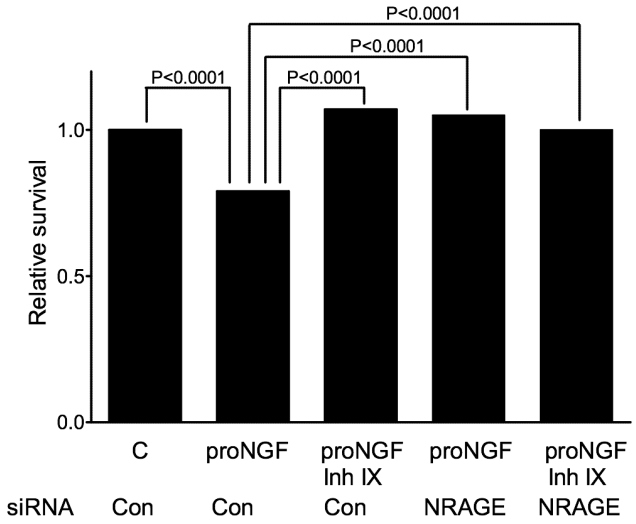
Figure 3.14: Effect of NRAGE siRNA on survival of neo-natal and aged SCG neurons

In neonates, NRAGE siRNA (80nM) significantly increased the survival of SCG neurons after NGF withdrawal ($p<0.0001$;$n=300$). Survival in the presence of NGF compared to NRAGE siRNA was not significantly different. In aged mice, NRAGE siRNA had no significant effect on the survival of NGF-treated SCG neurons ($p=0.50$; $n=900$)

NRAGE siRNA was subsequently administered in proNGF-treated SCG cultures of old mice. NRAGE siRNA knockdown significantly improved the survival of proNGF-treated aged SCG neurons, whereas neuritogenesis was not affected by NRAGE siRNA. When NRAGE siRNA and **JNK inhibitor IX** were added together, there was no significant change in aged SCG neuronal survival compared to those treated with **JNK inhibitor IX** alone. However, the effect of **JNK inhibitor IX** superseded the effect of NRAGE knockdown on neurite outgrowth, and proNGF-mediated growth signalling was completely abolished in aged SCG neurons (**Fig. 3.15**). Similar results were observed using **JNK Inhibitor VIII** (**Fig 3.16**) although, as observed throughout these studies, there was a less pronounced effect on neurite growth with this inhibitor compared to **JNK Inhibitor IX.**

Overall these results suggest that NRAGE is only involved in survival signalling, under these conditions, and is not implicated in the proNGF-mediated and JNK-dependent neurite outgrowth of aged SCG neurons.

. **A**



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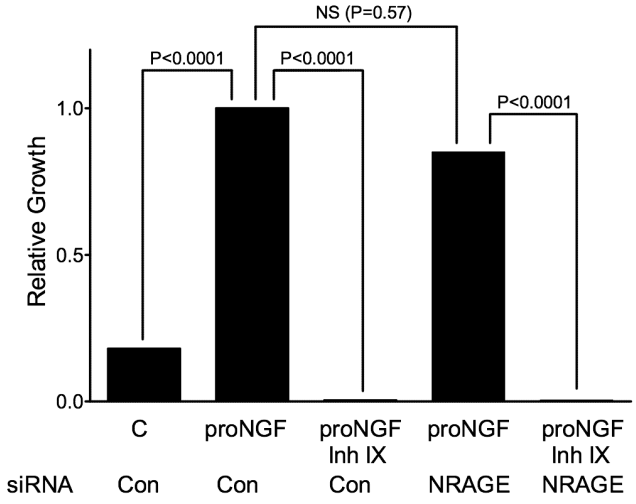
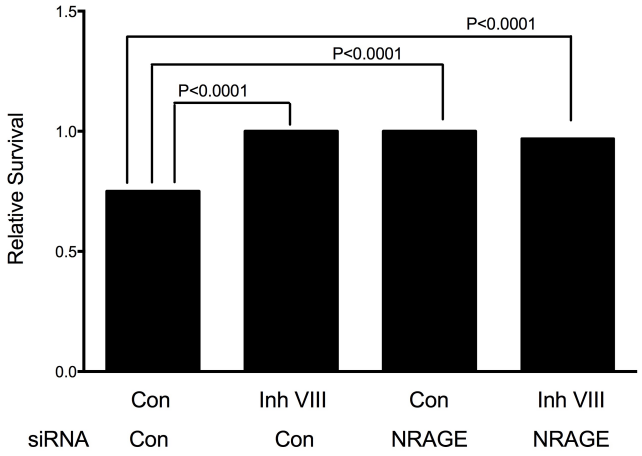


Figure 3.15: Effect of JNK inhibitor IX and NRAGE siRNA on proNGF-induced cell death and neurite outgrowth of aged SCG neurons

On Day 1, (A) JNK Inhibitor IX and NRAGE siRNA significantly reduced the proNGF-mediated cell death of aged SCG neurons ($p<0.0001$). (B) NRAGE siRNA had no effect on neurite outgrowth of proNGF-treated aged neurons ($p<0.0001$). JNK Inhibitor IX significantly abolished all neurite out growth of proNGF-treated neurons, also in the presence of NRAGE siRNA ($p<0.0001$)($n = 900$)

A



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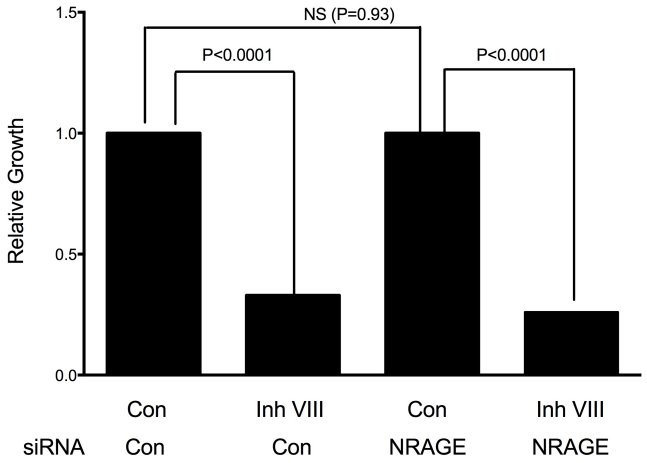

Figure 3.16: Effect of JNK inhibitor VIII and NRAGE siRNA on proNGF-induced cell death and neurite outgrowth of aged SCG neurons

On Day 1, (A) JNK Inhibitor VIII and NRAGE siRNA significantly enhanced the survival of aged SCG neurons, relative to control, in proNGF-treated cultures, ($p<0.0001$). (B) NRAGE siRNA had no effect on the neurite outgrowth of proNGF-treated aged SCG neurons ($p<0.0001$). However, JNK Inhibitor VIII significantly reduced the proportion of aged SCG neurons with neurite outgrowth in proNGF-treated culture, also in the presence of NRAGE siRNA ($p<0.0001$)($n = 900$).

To assess proNGF receptor levels, the expression or TrkA, Sortilin and NRAGE were determined by western blot analysis on SCGs from young adult and aged mice (**Fig 3.16**). No significant differences were observed in all three protein levels between young adult and old mice, when normalised against actin expression.

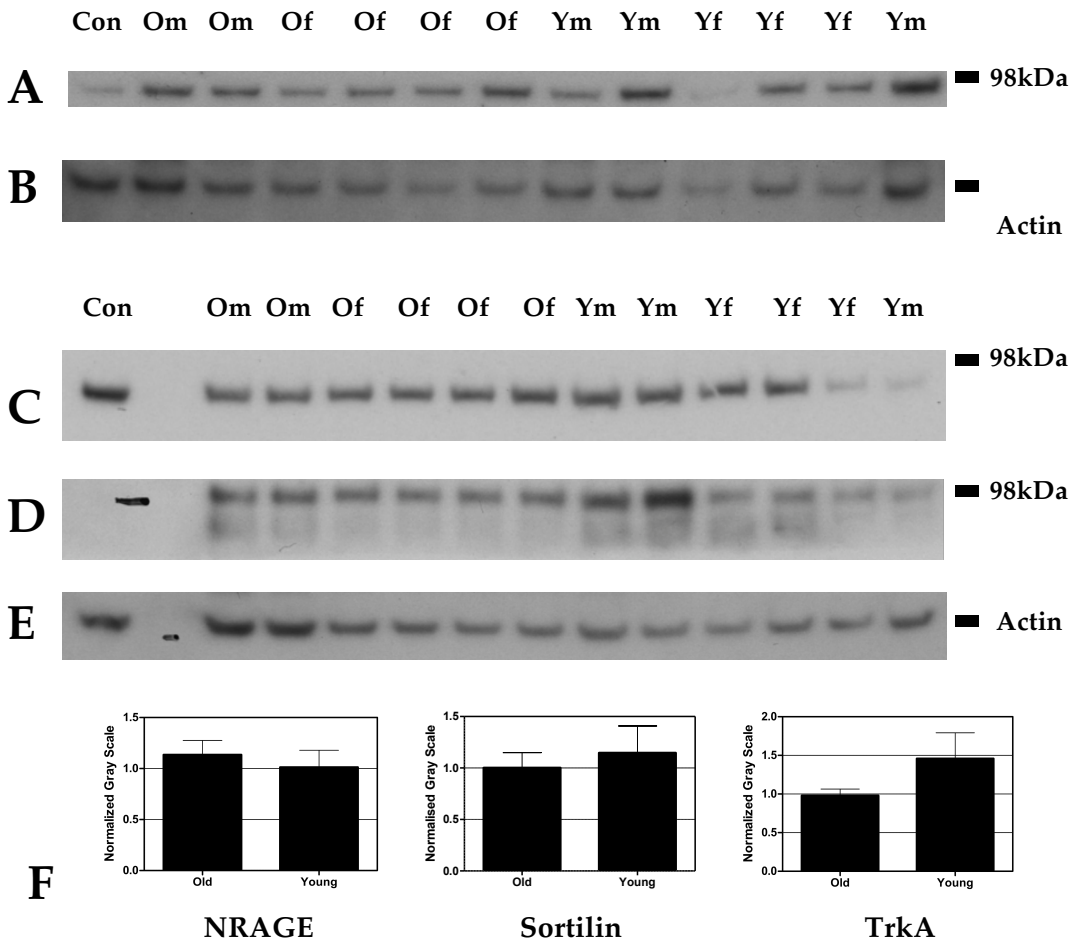

Figure 3.17: Western blot analysis of SCG lysates of and young adult mice

Each lane represents a pooled sample of six SCGs taken from three mice (A) NRAGE 90-95kDa, detected in the brain, p=0.59. (B) Actin relating to protein concentrations in A (C) Sortilin, 100kDa (D) TrkA, 98kDa not detected in the brain, p=0.24. E) Actin relating to protein concentrations in C/D. (F) No significant difference was observed between age-groups when normalised against actin expression for each protein. For each age-group, n=6, non-parametric t-test (Mann-Whitney). SCG samples are represented by Om – males, Of - females, Ym – Young males, Yf - Young females. Con – brain tissue.

3.3.4 Discussion

The foremost finding in this section is that JNK signalling is involved in both the proNGF-mediated cell death and neuritogenesis of cultured SCG neurons derived from old mice. JNK inhibition in aged SCG neuron cultures, through **AEG3482**, **JNK Inhibitor VIII and IX,** significantly rescued neurons from proNGF-mediated cell death as well as halting the growth of surviving neurons.

The $p75^{NTR}$ adaptor protein, NRAGE, was also found to be involved in proNGF-mediated cell death; siRNA knockdown of NRAGE increased the survival of proNGF-treated aged neurons in culture. This is in contrast to NGF control-treated cultures, where knockdown of NRAGE does not affect survival, and is consistent with earlier studies that aged neurons are not dependent on NGF for survival. While there have been reports of a role for NRAGE in neurite outgrowth, siRNA knockdown did not affect the neuritogenesis of cultured aged SCG neurons. Therefore, it is concluded that NRAGE is predominantly an important mediator of cell death in both neonatal and ageing sympathetic neurons.

Again, it was found that **JNK inhibitor IX** completely eliminates any neurite outgrowth, this time in the presence of proNGF in aged SCG neuron cultures, while **JNK inhibitor VIII** had less restriction. Similar to results in the first section, this indicates an involvement of JNK3 in promoting neurite outgrowth of surviving proNGF-treated aged sympathetic neurons.

JNK activation is associated with both regeneration and neuronal cell death, two contrasting pathways, which partly explains why neurons do not undergo extensive proNGF-mediated apoptosis *in vivo*. As discussed earlier (**Results, 3.3.1**), culture conditions may determine the level of proNGF-mediated neurotoxicity in cell culture systems. In the absence of insulin in the cell culture medium, it was found that proNGF was neurotoxic, whereas there was no significant effect of proNGF in cultures containing insulin, suggesting that insulin was having a neuroprotective effect on the survival of aged sympathetic neurons.

Similar to observations in aged rodent SCG neuronal culture studies conducted by (Al-Shawi et al., 2008), only a proportion of neurons were killed by proNGF. SCG are

composed of sub-populations of neurons, which project to different targets e.g. cerebral blood vessel (CV)-projecting and iris-projecting. Al-Shawi et al., (2008) found that in the CV of young adult rodents, proNGF levels were much higher than in the iris, but this was regulated in ageing where similar levels of proNGF were observed in both targets. Levels of proNGF studied by Bierl and Isaacson (2007) in SCG and peripheral targets from young adult rodents also show a degree of variability. This may contribute to the differing vulnerability in sub-populations of NGF-responsive neurons in ageing. Additionally, sub-types may contribute to the antagonistic effects on survival and growth observed in culture experiments through their complement of cell surface receptors. Immunohistochemistry studies on fixed cultures, soon after the addition of various JNK inhibitors, would help to identify how the complement of cell surface receptors is affected in these experiments.

In SCG lysates, western blot data revealed there was no difference in the levels of TrkA, sortilin and NRAGE levels in young adult compared with aged mice. TrkA levels were consistent with previous studies in SCG, where TrkA expression remains unchanged in mature adulthood, and old age (Cowen et al., 2003). However, the expression of sortilin was different to what has been previously observed. An increase in sortilin levels was previously reported in aged rat SCG lysates in comparison with young adult lysates and, in the same study, a stronger staining intensity was clearly visualised in immunohistochemical studies in aged mouse SCG compared to young adult (Al-Shawi et al., 2008). While sortilin levels may be variable across different species, the up-regulation in sortilin reactivity in ageing may be due to a change in the intracellular localisation of sortilin; sortilin is known to be recruited to the surface to influence pro-NGF mediated cell death. Variability has also been previously observed in NRAGE expression in the brain; there are higher levels of NRAGE in the cortex of aged mouse lysates compared to young adult, however there is no significant difference in the hippocampus (R. Al-Shawi; J.P Simons, personal communication). In this study it was found that levels do not change in SCG neurons of young adult and aged mice. The difference could be due to the fact that pooled samples were used for SCG samples whereas brain lysates were obtained from individual rodents. In any case, these results suggest that NRAGE expression is highly variable in the adult nervous system and may be differentially and developmentally regulated.

The JNK-mediated apoptotic signal in developing sympathetic neurons, following NGF withdrawal, involves phosphorylation of c-Jun (Estus et al., 1994; Ham et al., 1995; Eilers et al., 1998; Bruckner et al., 2001), or a number of other downstream effectors (Tapon et al., 1998; Xu et al., 2003; Kristiansen et al., 2011). While this study confirms that proNGF-mediated cell death in aged sympathetic neurons is JNK- and NRAGE- dependent, the downstream nuclear pathway involved in this process is still unknown. Therefore, further studies are required to fully characterise this molecular signalling pathway. Similarly, the specific downstream targets involved in the process of proNGF/JNK mediated neurite outgrowth are yet to be determined.

It has been suggested that distinct outcomes of positive and negative influences on neuronal behaviour may be down to opposing functions of nuclear and cytosolic JNK (Bjorkblom et al., 2008). The cytosolic pool of activated JNK promotes neurite outgrowth, through modulation of cytoskeletal proteins (Coffey et al., 2000; Chang et al., 2003; Gdalyahu et al., 2004; Tararuk et al., 2006). Therefore the action of neuritogenesis in proNGF-treated cultures, which is restricted by the introduction of JNK inhibitors, could be mediated by JNK in the cytosol but further immunostaining or radiolabeling would be required to confirm this.

Chapter 4
- GENERAL DISCUSSION -

4.1 JNK-dependent neurotrophic signalling

In ageing, neuronal loss is observed in peripheral systems alongside slowing rates of regeneration in response to injury. Levels of neurotrophic factors and receptor localisation all play their part but the role of downstream effectors is less clear. While reports have highlighted the involvement of the JNK family in neurotrophic signalling during neurodegeneration, and age-related diseases, much is yet to be discovered in the PNS. The work presented in this thesis establishes a role for JNK in neurotrophin-responsive young adult and aged sympathetic neurons.

JNK inhibitor studies on cultured NGF-treated SCG neurons revealed that JNK has a fundamental role in facilitating the regrowth of aged SCG neurons but does not have an impact on survival. Therefore, while JNK is involved in the removal of neurons unable to sustain neurotropic support in early development, this study is consistent with the notion that aged SCG neurons are not dependent on NGF for survival (Orike et al., 2001b; Al-Shawi et al., 2008). Similar results were found in cultured young adult SCG neurons suggesting that adult age does not have an impact on the neuronal response to JNK inhibition during NGF signalling.

In line with previous work, proNGF mediates cell death, as well as neuritogenesis of aged SCG neuron cultures. Here, it was demonstrated that JNK assists with proNGF-induced growth of adult and aged sympathetic neurons and significantly, JNK inhibition rescues neurons from proNGF-mediated cell death. Hence, it appears the importance of JNK in survival only manifests itself when a sub-population of neurons are subjected to a stimulus promoting cell-death.

We know that localised areas of high proNGF and sortilin levels enhance cell death in vulnerable populations of basal forebrain neurons of the CNS, and populations of sympathetic neurons of the SCG in aged rodents (Bierl and Isaacson 2007; Al-Shawi et al., 2008). Further studies are necessary to determine whether any particular sub-population of sympathetic neurons is more susceptible to proNGF/JNK-mediated cell death.

4.1.1 Pan JNK-inhibition

The findings described above are based on pan-JNK inhibitor studies, whereby all three JNK isoforms are inhibited. The common use of non-specific small-molecule inhibitors of JNKs can add to the challenge of identifying which MAPK, JNK or p38, exerts a given function. However, the pan-JNK inhibitors used in this study have been tried and tested in previous experiments and display a high specificity for JNK (Kita et al., 1998; Bennett et al., 2001; Holzberg et al., 2003; Lindwall and Kanje 2005). Further use of isoform-specific inhibitors (**4.1.2**), which display even greater specificity for JNK (Szczepankiewicz et al., 2006; Angell et al., 2007), were able to support results following the use of pan-JNK inhibitors.

JNK inhibitor II works by competitive inhibition of the JNK ATP binding site and **JNK inhibitor III** specifically disrupts the c-Jun/JNK complex formation. Despite possessing alternate modes of inhibition, the same outcome was achieved in the presence of NGF-treated SCG cultures (neurite outgrowth restriction and no effect on the survival of aged sympathetic neurons). **AEG3482** was also used in these experiments to inhibit JNK. Following the administration in NGF-treated young adult and aged SCG cultures, the same overall outcome was induced. **AEG3482** works to prevent neonatal NRAGE/JNK mediated cell death by binding to HSP90 and disrupting the association with heat shock protein transcription factor HSF-1. Freely available HSF-1 subsequently induces the expression of HSP70, which is known to block JNK activation (Salehi et al., 2006). In this study, the survival of cultured aged SCG neurons was not affected by the addition of AEG3482, however an effect on a small population of neurons cannot be ruled out. Western and immunohistochemical studies supported this idea, and even though there was no significant difference in HSP70 levels of aged neurons cultured with AEG3482, the standard error of mean of HSP70 staining intensities, when normalised against control, was extremely high. Therefore, the differing vulnerability of SCG neuron subpopulations may also be a consideration here. The involvement of HSP70 has also been identified in the process of neurite outgrowth. The HSP70 inhibitor, KNK437 enhances neurite outgrowth in PC12 cells. As there was no significant up-regulation in HSP70 levels in aged neurons, it may be that AEG3482 is restricting growth via a different pathway. Another possibility is that HSP70 levels were elevated but this was not visualised.

The impact on survival in proNGF cultures proved to be more obvious and JNK inhibition through **AEG3482** rescued aged SCG neurons subjected to proNGF-mediated cell death. Whether this occurred through **AEG3428**-induced up-regulation of heat shock proteins remains to be investigated. Similarly, it was quite apparent that the growth of aged SCG neurons was severely impaired by **AEG3482** and further work is required to determine the pathway by which **AEG3482** influences this reduction.

4.1.2 JNK Isoforms

Previous research indicates that JNK isoforms may have specialised roles within the cell, something that is illustrated in the kainic-acid (excitotoxic glutamate receptor agonist) induced injury model of adult transgenic mice. In JNK1 KOs, increased neuronal degeneration compared to wild-type mice is observed (along with a tendency for enhanced seizures), JNK2 KOs are unaffected, and JNK3 KOs attenuated seizures and decreased neurodegeneration (Brecht et al., 2005). This, combined with the idea that improved neuroprotection could be achieved through selective inhibition of JNK isoforms has powered the search for isoform-specific inhibitors, particularly for JNK2 and JNK3 isoforms, which clearly display pro-death properties in neurons of the adult brain (Yang et al., 1997; Coffey et al., 2002; Brecht et al., 2005).

Previous studies implicate JNK3 in the p75-mediated apoptosis of neonatal sympathetic neurons after NGF withdrawal (Bruckner et al., 2001; Kenchappa et al., 2010). This thesis also highlights the importance of JNK3 in proNGF-treated aged SCG neurons cultures. **JNK Inhibitor IX (**inhibits JNK2 and JNK3) was more effective than **JNK Inhibitor VIII** (inhibits JNKI and JNK2 more effectively than JNK3) in rescuing cultured aged SCG neurons from proNGF-mediated cell death so JNK3 is likely to be important in proNGF-mediated signalling in aged neurons.

Experiments on JNK actions on neurite outgrowth have also been performed but largely in neuronal cell lines such as PC12 cells (Heasley et al., 1996). When undertaking research, it is important to note that not all JNK isoforms are expressed by these cells. While JNK1 and 2 have been shown to induce neurite outgrowth, JNK3 is not expressed in PC12 cells and requires exogenous transfection to induce NGF-induced sprouting (Waetzig and Herdegen 2003b). In this study, the

administration of different isoform-specific JNK inhibitors suggest JNK3 to be a more effective regulator than JNK1/2 for neuronal outgrowth of cultured aged SCG neurons in the presence of NGF and proNGF. However, further inhibitor studies are required to determine the specificity. In peripheral axons, JNK3 forms a complex with kinesin 1, JIP3 or Syd (a mammalian homolog of JIP3), which mediates the axonal transport of JNK by interaction with the dynactin/dynein motor system (Cavalli et al., 2005). Following axotomy of mouse sciatic nerves, JNK3 is activated and anterograde transport is disrupted, whereas retrograde transport increases. It is believed that JNK3 acts as a surveillance sensor that responds to stress by altering motor transport.

To complement this study, research into adult DRG found that neuritogenesis is delayed by lack of JNK2 and JNK3, but not JNK1 (Barnat et al., 2010). However, Barnat et al. (2010) goes on to show that JNK signalling is further required for sustained neurite elongation as pharmalogical pan-JNK inhibition resulted in neurite retraction, which relies on JNK1 and 2. Studies indicate that JNK1 could have a fundamental role in elongation by phosphorylating proteins that regulate microtubule dynamics, such as MAP1B, MAP2, and stathmin family proteins (Chang et al., 2003; Bjorkblom et al., 2005; Tararuk et al., 2006). This was not investigated in this study as JNK inhibitors were administered at the time of plating but there is potential for further studies on the impact of JNK inhibition on elongation after neurite outgrowth has been initiated.

4.1.3 $p75^{NTR}$ adaptor protein NRAGE

Once activated, the $p75^{NTR}$ receptor is dependent on the recruitment of adaptor protein complexes for further signalling, due to a lack of intrinsic catalytic activity. In NGF-responsive neurons, the $p75^{NTR}$/NRAGE interaction is involved in JNK-mediated apoptosis (Salehi et al., 2000).

The results in this thesis suggest that NRAGE helps to mediate cell death at all stages of life, from early development to ageing. Significantly, this was the first report of NRAGE involvement in neurotrophic signalling of aged SCG neurons and, similar to

JNK inhibition, NRAGE knockdown only rescued survival during proNGF-induced cell death. Survival of neurons remained unaffected in the presence of NGF.

It was also revealed that neuritogenesis of aged SCG neurons was unaffected after NRAGE knockdown, which supports previous NRAGE knockout studies in mice where neuronal growth was unaffected (Bertrand et al., 2008). This, however, differs from results observed in PC12 cells. Reddy et al, 2010 reported that expressing endogenous NRAGE enhanced NGF-dependent neurite outgrowth, while Feng et al., 2010, found that NGF-mediated outgrowth is accelerated following RNA knockdown of NRAGE. Therefore, although NRAGE has been linked with $p75^{NTR}$-dependent cell cycle arrest in sympathetic neurons (Kendall et al., 2002; Reddy et al., 2010), there is still some uncertainty as to the impact of NRAGE on neuritogenesis.

As NRAGE expression is predominantly confined to neurons (Barrett et al., 2005), western blot analysis gives suitable reflection of protein levels in neurons from aged tissue samples, which contain a combination of cell types. Low NRAGE expression is found in the adult nervous system, (Salehi et al., 2000; Kendall et al., 2002), and there is substantial variation in NRAGE expression levels between individuals, and between species in different regions of the adult nervous system (R. Al-Shawi; J.P Simons, personal communication). Previous SCG lysates from individual rats, and lysates from pooled mouse SCG in this study, show considerable variation between samples.

In addition, this study observed no significant difference between young adult or aged animal samples of pooled sympathetic neurons, when normalised against actin expression. However, studies in the mouse brain revealed that in some regions vulnerable to age-related neuronal loss, NRAGE protein expression is elevated on ageing, although these samples were taken from individual mice (R. Al-Shawi; J.P Simons, personal communication). In any case, this local and regional variability observed between individuals suggests that NRAGE expression is highly plastic in the adult nervous system.

4.1.4 Glia

More attention is frequently placed on neurons in research, and glial responses have not been as thoroughly investigated. In the PNS, JNKs control cell proliferation and death of Schwann cells, and have also been implicated in Schwann cell de-differentiation during the process of nerve regeneration (Monje et al., 2010). A better understanding of the delicate mechanisms behind the survival and proliferation in glial cells, and the switch into a regenerative state in neurons is an essential part in our efforts to improve the outcome of nerve repair, post nerve injury and during ageing.

In this study, similar to neurons, JNK inhibitors halted the proliferation of glia isolated from the SCGs of aged adult mice. The growth of glial extensions was also restricted to varying degrees depending on the type of inhibitor. **JNK inhibitor IX** was, again, the most effective inhibitor. After following the cultures for three days, the glia appeared to shrivel up but Live/Dead assays confirmed that glia were still alive.

The association of JNKs in the regulation of proliferative and terminally differentiated oligodendrocytes, in cell death and disease also exists in the CNS (Casaccia-Bonnefil et al., 1996; Ladiwala et al., 1998; Yoon et al., 1998; Jurewicz et al., 2003; Gadea et al., 2008) and in response to injury (Gadea et al., 2008). In most cases, the proliferation or hypertrophy of several different types of glial cells, including oligodendrocytes in response to injury can lead to abnormal levels of glia resulting in gliosis (Robel et al., 2011). In turn, this can be detrimental to nearby neurons. Glial scar formation due to reactive astrogliosis in spinal cord injury can also be detrimental as it forms an impenetrable barrier for regenerating axons (Yuan and He 2013). The results in this study may have therapeutic implications in the use of JNK inhibitors for regulating and inhibiting these reactions, without killing the glia and surrounding neurons.

It would also be of interest to fully characterise the population and composition of specific glia post-injury in adult SCG and during ageing. As satellite glial cells and Schwann cells display different characteristics and possess different biological functions, it may be that they respond differently to drug application and this might directly or indirectly affect their relationship with neurons.

4.1.5 Implications for treatment

Realistically, the only way to currently evaluate the importance of experimental findings is to develop, and test, a given treatment in human subjects. While the conversion of experimental findings to the clinic is possible, it is not always a clear-cut process. There are plenty of examples of encouraging preliminary results in animals that fail to materialise into clinically effective compounds. However, assembling evidence from clinically relevant studies does have the ability to further support data from animal experiments.

A suggestion for the use of JNK inhibitors during the process of gliosis has already been discussed but there may also be implications for neuronal pathologies. In Alzheimer's disease, retrograde transport of NGF from the cortex and hippocampus to basal forebrain cholinergic neurons (BFCN) is reduced as these neurons degenerate, with the simultaneous accumulation of proNGF in the cortex and hippocampus (Fahnestock et al., 2001; Peng et al., 2004; Pedraza et al., 2005). Interestingly, post-mortem brains from patients with this disease have been shown to display distinctly high levels of JNK activity as well (Ferrer et al., 2001). Although studies in AD have primarily focused on the role of JNK in increasing amyloid-β plaque load (Mazzitelli et al., 2011), and hyper-phosphorylated tau (Reynolds et al., 1997; Reynolds et al., 2000), the relevance of JNK and the association with proNGF-mediated cell death may be important in this vicinity too.

JNK1 and JNK3 present distinct subcellular distributions (Lee et al., 1999; Brecht et al., 2005) (Lee et al., 1999) in areas of the adult mouse brain. JNK3 is prominent in the nuclei of a subset of neurons (~30%) in the cortex and JNK1 expression dominates in the somal cytoplasmic space and neurofibres. In the hippocampus, JNK3 expression is found in ~90% of pyramidal layer neurons, whereas JNK1 expression is restricted to areas of the dentate gyrus (Lee et al., 1999). It would certainly warrant further investigation to observe whether proNGF-mediated cell death in an AD model is not only JNK-dependent, but isoform-specific, and if subcellular distribution has an impact on this.

4.1.6 Culturing aged neurons

In culture conditions, neurons are taken out of an *in vivo* environment and placed in an artificial one. The administration of collagenase and trypsin, and the process of trituration isolates the neurons, strips them from cell adhesion molecules and places them under a considerable amount of stress. It is inevitable that they seek to enter a state of regeneration once placed in growing conditions. In this study, it appears that aged neurons are more robust than neonatal neurons. In fact, aged and young adult neuronal cultures were able to last for three days without changing the medium. Because of this, cells could be followed for a number of days to observe the development of various signalling pathways. An interesting observation was the breakdown of neuronal cultures, sometimes by Day 3, after a high percentage of initial neurite outgrowth. This was taken into account during analysis but something to keep in mind for further experimentation. Although refreshing the medium would mean the disturbance of aged neurons, their robust nature might allow them to withstand this disruption and help cultures thrive longer than we initially envisaged. On the whole, this study validates previous experiments used for culturing aged neurons (Orike et al., 2001a; Al-Shawi et al., 2008), and provides further evidence of a reliable model for research of aged sympathetic neurons.

It also provides a suitable model to investigate pro-regenerative responses in ageing, something that has traditionally been examined *in vivo*, following conditioning lesions or drug application, mostly in the DRG and sciatic nerve of young adult rodents (McQuarrie and Grafstein 1973; Kilmer and Carlsen 1984; Smith and Skene 1997; Neumann et al., 2002; Qiu et al., 2002). As discussed in the **General Introduction**, only recently has it been established that axotomy of peripheral sensory neurons (DRG), by dissociating and culturing them, prompts a similar response to *in vivo* conditioning lesions. The activation of injury-associated transcription factors and up-regulation of regeneration-associated genes (Smith and Skene 1997; Saijilafu et al., 2013), followed by the characteristic neurite regrowth patterns occur within the first 24 hours of culture (Frey et al., 2015). Supplementary to the work described in this thesis, Frey et al. (2015) continued to demonstrate that stimulation of the pro-regenerative state *in vitro* requires JNK signaling, a key factor within the pro-regeneration response *in vivo*. The group also identified an *in vitro* platform to induce a pro-regenerative state, in the absence of injury, by guiding neurons into a

''functionally naïve'' state. Thus creating an assay capable of selectively identifying drugs that induce the pro-regenerative state, rather than directly modulating neurite extension.

Understanding the mechanisms responsible for inducing the pro-regenerative program could have important consequences for axon regeneration in ageing and a variety of neurological disorders. Based on the results of this study, the use of JNK inhibitors to assist with peripheral nerve regeneration would perhaps not be advised. However, there is potential for transferring the Frey et al. (2015) model to aged sympathetic neurons and administering JNK inhibitors to more closely identify the role of different isoforms in an enhanced pro-regenerative state similar to *in vivo*. It would also help to complement the search for more specialised JNK inhibitors that replicate actions *in vivo.*

4.1.7 Future Directions

Despite the suggestions above, the benefits of acquiring *in vivo* information on the influence of JNK post injury and during ageing, in sympathetic neurons, must not be forgotten. Reviews by Bogoyevitch et al. (2010) and Coffey et al. (2014) give detailed accounts of JNK KO studies to date. From the evidence provided, the oldest model previously used for JNK knockout studies are 8-month old mice (Chen et al., 2001; Chang et al., 2003). Chang et al., used *Jnk1*$^{-/-}$ KOs to observe the impact on the structural maintenance of microtubules in the spinal cord and hippocampus, while Chen discovered an effect of *Jnk2*$^{-/-}$ KOs on skin tumorogenesis.

The lack of JNK knockout studies in ageing research may be attributed to earlier fatalities or the costs of breeding animals for extended periods. While there is limited data on aged rodent knockouts, there is plenty of information during early development and to a lesser extent, in adults. Only compound mutants lacking *Jnk1* and *Jnk2* genes together were embryonically lethal (Kuan et al., 1999) suggesting that there is adequate scope for other JNK isoform knockout studies. Furthermore, JNK studies in the context of axotomy and regeneration are also deficient beyond the regenerative role of c-Jun, in the adult rat, following facial nerve fibre cut (Ruff et al., 2012). So, an important extension of the work described in this thesis would be to

implement these *in vivo* JNK knockout models in aged rodents, and post injury, to complement results from cell culture methods. Inducible methods would be especially useful to differentiate between developmental and ageing effects.

Ultimately, there is still a huge cloud over the outcome of neurotrophic signalling in ageing, and after injury. The dichotomy of neuritogenesis and cell death quite clearly exists and vital factors along the chain perfectly symbolise this. Although it is necessary to fully characterise which activators and effectors steer JNK signalling in a particular direction, other key players require attention.

4.1.8 Future areas of study and new emerging therapies

In recent years, studies have indicated that the $p75^{NTR}$ signalling receptor is part of a regulatory program to remove defective neurons and axons post injury and during degeneration in adults, with a propensity to worsen the damage by the formation of severe lesions. As a result, small $p75^{NTR}$-binding peptides (Longo et al., 1997) and organic compounds (Massa et al., 2006) have been generated to counteract this. Ibanez and Simi (2012) give a good account of these, as well as the expression and functional consequence of $p75^{NTR}$ signalling after neural injury, and cellular stress. Although $p75^{NTR}$ has been shown to promote cell-death, it also has to ability to influence cell survival (**Introduction**).

A suggested role for NRAGE and other $p75^{NTR}$ interacting proteins as future drug targets, certainly in $p75^{NTR}$/JNK-mediated apoptosis, has increased their value in ongoing research. However, information on the changes in the expression of other $p75^{NTR}$ interacting proteins e.g. NRIF, is still lacking during ageing. In neuritogenesis, the question of which scaffold proteins are also involved requires further clarity. Previous findings suggest the possible involvement of JIP-1 and JIP3 in the organisation of JNK-mediated microtubule dynamics underlying neurite outgrowth but studies have been limited to PC12 cells (Meyer et al., 1999; Sato et al., 2004).

While results displayed the involvement of JNK in proNGF-mediated cell death and neuritogenesis, further studies would help to confirm how this arises further downstream. The expression of transcription factors, such as c-Jun, activator protein 1 (AP-1) and other downstream targets are also important regulators of signalling

pathways e.g. c-Jun, which is involved in a myriad of responses such as neuritogenesis, regeneration and axotomy-induced death in the adult nervous system, and is not just phosphorylated by JNK thus adding to the complexity of the system

Another inviting direction for prospective research is to delve deeper into the impact of subcellular localisation of specific JNK isoforms. A report by Bjorkblom et al., (2008) in the brain challenged the idea that inhibition of a single JNK isoform (for example, JNK3) provides neuroprotection in adults and older subjects and suggested that subcellular compartment-specific targeting was more effective.

It was previously reported in cerebellar granule neurons, that while JNK1 is present mainly in the cytoplasm of neuronal processes, JNK2 and JNK3 can be translocated to the nucleus and affect transcription of regeneration-associated genes e.g. c-Jun (Coffey et al., 2002). With this in mind, targeted inhibitors of JNK were created, by fusing a nuclear export sequence (NES) or a nuclear localisation sequence (NLS) upstream of the JNK inhibitor JBD resulting in constructs with selectivity of action in the cytosol and nucleus (Bjorkblom et al., 2005; Tararuk et al., 2006). Constructs expressed in cerebellar granule neurons of neonatal rats, with selectivity of JNK inhibition in the cytosol, imitates the *Jnk1* KO study (Coffey et al., 2002) by causing radial migration in the developing brain and a reduction in neurite growth. By contrast, nucleus selective JNK inhibitor constructs do not disrupt these physiological events (Bjorkblom et al., 2005; Tararuk et al., 2006) but they do provide substantial protection from $p75^{NTR}$ induced neuronal death in response to trophic support withdrawal (Bjorkblom et al., 2008). This is supported by recent studies in HEK293 cells where JNK activity prevents $p75^{NTR}$ receptor cell death signalling in the nucleus but not the cytoplasm (Charalampopoulos et al., 2012). Essentially, the report demonstrates the importance of nuclear JNK for signalling death and cytoplasmic JNK for neurite outgrowth.

Specific inhibition of JNK in mitochondria is another successful targeting approach. An enrichment of JNK in the mitochondria is visible following excitotoxic stresses e.g. transient middle cerebral artery occlusion and kainate treatment in the brain (Zhao and Herdegen 2009). A peptide developed by Chambers et al., (2011) which blocks JNK recruitment to mitochondria, reduces ischemic damage in neonatal rats (Nijboer et al., 2013). Further information on drug therapy and compartment-specific

targeting of JNK to block pro-apoptotic JNK action has been reviewed by Coffey (2014). The possibility for organelle targeting by peptide drugs has certainly unraveled a promising therapeutic strategy (Graczyk 2013). It is definitely an area that would help to complement the work in this thesis and in existing and current literature.

4.1.9 Closing remarks

Throughout life, cells are differentially regulated depending on their requirement at different stages of development, maturation and regeneration. Environmental cues, receptor complement expression and the complex temporal and spatial regulation of relevant ligands and intracellular signalling proteins all contribute to the desired signalling outcome. Quite clearly, there are a number of factors at work during the process of ageing and we are far from predicting the final outcome of signalling pathways because of increased variability. However, a better grasp of the components and the machinery that make up these molecular pathways will serve to enhance our understanding and also help to provide better strategies for the effective treatment of neurodegeneration.

Chapter 5
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